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MULTI-ARRAY, MULTI- SPECIFIC ELECTROCHEMILUMINESCENCE TESTING,
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MULTI-ARRAY, MULTI-SPECIFIC
ELECTROCHEMILUMINESCENCE TESTING

This application claims priority to provisional application 60/012,958, filed March 6, 1996 and is a continuation-in-part application of copending application Serial No. 08/402,076 filed March 10, 1995 and of copending application Serial No. 08/402,277 filed March 10, 1995, each of which is incorporated by reference herein in its entirety.

1. INTRODUCTION

The present invention provides for a patterned multi-array, multi-specific surface (PMAMS) for electrochemiluminescence based tests, as well as methods for making and using PMAMS.

2. BACKGROUND OF THE INVENTION

2.1. DIAGNOSTIC ASSAYS

There is a strong economic need for rapid sensitive diagnostic technologies. Diagnostic technologies are important in a wide variety of economic markets including health care, research, agricultural, veterinary, and industrial marketplaces. An improvement in sensitivity, time required, ease of use, robustness, or cost can open entirely new diagnostic markets where previously no technology could meet the market need. Certain diagnostic technologies may possess high sensitivity but are too expensive to meet market needs. Other techniques may be cost effective but not robust enough for various markets. A novel diagnostic technique which is capable of combining these qualities is a significant advance and opportunity in the diagnostics business.

There are a number of different analytical techniques used in diagnostic applications. These techniques include radioactive labeling, enzyme linked immunoassays, chemical colorimetric assays, fluorescence labeling, chemiluminescent labeling, and electrochemiluminescent labeling. Each of these techniques has a unique combination of sensitivity levels, ease of use, robustness, speed and cost which define and limit their utility in different diagnostic markets. These differences are

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in part due to the physical constraints inherent to each technique. Radioactive labeling, for example, is inherently non-robust because the label itself decays and the disposal of the resulting radioactive waste results in economic, safety and environmental costs for many applications.

Many of the sensitive diagnostic techniques in use today are market-limited primarily because of the need for skilled technicians to perform the tests. Electrochemiluminescent procedures in use today, for example, require not only skilled technicians but repeated washing and preparatory steps. This increases both the costs and the need for waste disposal. Novel diagnostics which simplify the testing procedures as well as decrease the cost per test will be of great importance and utility in opening new markets as well as improving performance in existing markets.

2.2. ELECTROCHEMILUMINESCENCE ASSAYS

Electrochemiluminescence ("ECL") is the phenomena whereby an electrically excited species emits a photon (see, e.g., Leland and Powell, 1990 *J. Electrochem. Soc.* 137(10):3127-3131). Such species are termed ECL labels and are also referred to herein as TAGs. Commonly used ECL labels include: organometallic compounds where the metal is from, for example, the noble metals of group VIII, including Ru-containing and Os-containing organometallic compounds such as the Ru(2,2'-bipyridine)³⁺ moiety (also referred to as "Rubpy"), disclosed, e.g., by Bard et al. (U.S. Patent No. 5,238,808). The light generated by ECL labels can be used as a reporter signal in diagnostic procedures (Bard et al., U.S. Patent No. 5,221,605). For instance, an ECL label can be covalently coupled to a binding agent such as an antibody or nucleic acid probe. The ECL label/binding agent complex can be used to assay for a variety of substances (Bard et al., U.S. Patent No. 5,238,808). Fundamental to ECL-based detection systems is the need for an electrical potential to excite the ECL label to emit a photon. An electrical potential waveform is applied to an ECL assay solution

across an electrode surface, typically a metal surface, and a counterelectrode (see e.g., U.S. Patent Nos. 5,068,088, 5,093,268, 5,061,445, 5,238,808, 5,147,806, 5,247,243, 5,296,191, 5,310,687, 5,221,605).

Various apparatus well known to the art are available for conducting and detecting ECL reactions. For example, Zhang et al. (U.S. Patent No. 5,324,457) discloses exemplary electrodes for use in electrochemical cells for conducting ECL. Levantis et al. (U.S. Patent No. 5,093,268) discloses electrochemical cells for use in conducting ECL reactions. Kamin et al. (U.S. Patent No. 5,147,806) discloses apparatus for conducting and detecting ECL reactions, including voltage control devices. Zoski et al. (U.S. Patent No. 5,061,445) discloses apparatus for conducting and detecting ECL reactions, including electrical potential waveform diagrams for eliciting ECL reactions, digital to analog converters, control apparatus, detection apparatus and methods for detecting current generated by an ECL reaction at the working electrode to provide feedback information to the electronic control apparatus.

The ECL technique is reviewed in detail by, for example, U.S. Patent No. 5,093,268. In brief, the ECL technique is a method of detecting in a volume of a sample an analyte of interest present in the sample in relatively small concentrations.

The ECL moiety, termed a TAG in the above referenced issued patents, may or may not be bound to an analyte, but in either case is promoted to an excited state as a result of a series of chemical reactions triggered by the electrical energy received from the working electrode. A molecule which promotes ECL of the TAG is advantageously provided, such as oxalate or, more preferably, tripropylamine (see U.S. Patent No. 5,310,687).

2.3. COMMERCIAL ECL ASSAYS

To date, all commercial ECL reactions are carried out on centimeter scale electrode surfaces. The centimeter scale electrodes strike a balance between the enhanced magnitude of an

ECL signal resulting from larger electrodes and the desirability of decreasing the total sample volume necessary for each assay. However, even centimeter scale electrodes fail to achieve the sensitivity required for many assays. In an attempt to overcome this problem, all commercial ECL systems further enhance sensitivity by using coated magnetic beads to capture ECL analytes or reagents. The beads are then moved adjacent to a working electrode for enhanced sensitivity.

However, the use of magnetic beads has many limitations. The beads themselves are coated with proteins which slough off and degrade over time, causing signal variations. Due to the complexity in handling and formatting bead-based assays, commercial ECL diagnostics require a complex, serially performed set of procedures for each assay conducted with a given sample that increases the time and cost for each test to be performed. The 5 micron scale of the beads prevents most of the bead-bound ECL TAG from reaching the thin film adjacent to the working electrodes, resulting in inefficiency in excitation of the ECL TAG.

Leventis et al. (U.S. Patent No. 5,093,268) has proposed a method of assaying more than one different analyte simultaneously by the use of different ECL labels for each analyte, each emitting photons at different wavelengths for each different analyte in a single assay. However, this technique is limited, for example, by the unavailability of a sufficient number of effective ECL labels radiating at different wavelengths and the need to optimize the chemical conditions for each ECL label. These practical constraints have prevented the commercialization of such multi-wavelength, multi-analyte ECL detection systems.

Another approach to increased ECL sensitivity is to improve the electrode technology. Zhang et al. (U.S. Patent No. 5,324,457) have directly deposited films of ECL species on various metal and semiconductor surfaces. The Zhang and Bard technique using bulk saturation of the electrode surface results

(as described by the authors) in an uneven patchy deposition unsuitable for highly sensitive assays.

The previous methods for conducting an ECL assay also requires that the assay cell, including the electrodes, must be cleaned by any one of a number of methods, including the use of dilute acids, dilute bases, detergent solutions, and so forth as disclosed, for example, by U.S. Patent No. 5,147,806.

It is therefore an object of the present invention to provide a novel and cost effective assay for conducting a plurality of ECL reactions, either sequentially or simultaneously and in a preferred embodiment, providing built-in control standards for improved accuracy.

It is a further object of the present invention to provide a cassette comprising one or more supports suitable for conducting a plurality of simultaneous or sequential ECL reactions that is also disposable.

It is a further and related object of this invention to reduce the time and cost of conducting individual assays for analytes of interest in biological samples.

It is still a further and related object of this invention to provide methods and apparatus for conducting a plurality of simultaneous assays for a plurality of analytes of interest in a single biological sample.

3. SUMMARY OF THE INVENTION

The invention relates to a cassette for conducting ECL reactions and assays comprising a plurality of discrete binding domains immobilized on a support, the discrete binding domains being spatially aligned with one or more electrode and one or more counterelectrode pairs. The cassette preferably includes a first support having a plurality of discrete binding domains immobilized on the surface. It may have one or more electrode and one or more counterelectrode pairs. The electrode and counterelectrode-pairs are separately addressable by a source of electrical energy in the form of a voltage waveform effective to trigger electrochemiluminescence. The cassette may also comprise

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a second support capable of being placed adjacent to the first support to provide sample containing means therebetween, and/or serve as an electrode. The binding domains are patterned on a support surface and are prepared so as to bind analytes or reagents of interest.

The invention further relates to an apparatus for measuring electrochemiluminescence of a sample that provides support or cassette handling means, voltage control means adapted to apply a controlled voltage waveform effective to trigger electrochemiluminescence, photon detector means for detecting electrochemiluminescence from the sample and sample handling means.

The invention further relates to methods for using the cassettes for measuring electrochemiluminescence in a sample by contacting the plurality of binding domains of a cassette with a sample which contains a plurality of analytes of interest, under ECL assay conditions, and then applying a voltage waveform effective to trigger electrochemiluminescence at each of the plurality of electrode and counterelectrode pairs and detecting or measuring of the triggered electrochemiluminescence. In a broad aspect, the invention provides ECL assay methods in which the sample does not contact an electrode. Additionally, as an alternative to the use of electrode and counterelectrode pairs, the invention provides for scanning an electrode and counterelectrode over the binding domains.

The invention also provides for kits comprising components including cassettes suitable for simultaneously measuring a plurality of electrochemiluminescence reactions, support surfaces and upon which a plurality of domains are immobilized assay, media for conduct of the ECL assay conducting chemical reactions.

The invention also provides electrodes prepared from graphitic nanotubes.

4. DESCRIPTION OF THE FIGURES

Figure 1 illustrates two supports forming a cassette according to the invention wherein a plurality of binding domains 14 are present on support 10 and a plurality of corresponding electrodes 16 is present on support 12 so that approximation of the supports places an electrode pair adjacent to each binding domain.

Figure 1A illustrates two supports forming a cassette according to the invention wherein a plurality of binding domains 14 are present on support 10 and a plurality of corresponding electrodes 16 is present on support 12 so that approximation of the supports places an electrode pair adjacent to each binding domain.

Figure 2 illustrates two supports forming a cassette according to the invention wherein a plurality of binding domains 30 on support 26 are adjacent to each of single electrodes 32 so that approximating supports 26 and 28 places each of counterelectrodes 38 adjacent to each of binding domains 30.

Figure 3 illustrates two supports forming a cassette according to the invention wherein a plurality of binding domains 48 have electrode counterelectrode pairs 50 adjacent thereto on support 44. Support 46 may optionally be placed adjacent to support 44 so that support 46 provides sample containing means adjacent to binding domains 48 and electrodes 50.

Figure 4 illustrates two supports forming a cassette according to the invention wherein a plurality of binding domains 64 on support 60 are contacted with a sample suspected of containing an analyte. Support 62 has regions 66 containing reaction medium for detecting or measuring an analyte of interest or for carrying out a desired reaction so that approximating support 60 and support 62 causes binding domains 64 and regions 66 to contact one another.

Figure 5A illustrates a top view of patterned binding domains for a multi-array, multi-specific binding surface. Geometric shapes, triangles, squares and circles, represent

binding domains specific for different analytes. The binding domains may be hydrophobic or hydrophilic. The surrounding surface may have the opposite property (hydrophilic or hydrophobic) of the binding domains to minimize spreading of binding reagents or analyte from the binding domains.

Figure 5B illustrates a top view of a microfluidics guide for delivering binding reagents and/or analytes to discrete binding domains. Each dot illustrates a cross section of a microfluidics guide (e.g., a capillary).

Figure 5C illustrates a side view of a microfluidics guide showing the approximation of registered or aligned microfluidic guides for delivering binding reagents and/or analytes to a multi array of patterned binding domains. Each microfluidic guide may deliver a different binding reagent to a discrete binding domain.

Figure 6A illustrates the approximation of a multi-array of electrodes in register with a surface having patterned multi-array, multi-specific binding domains. A removable electrode protection barrier is shown between the electrode array and the binding surface array. The entire assembly comprises a cassette for conducting a plurality of ECL reactions.

Figure 6B illustrates the approximation of an array of registered or aligned addressable working and counterelectrodes. The electrodes may be shape complementary with the binding domain or of other shapes (e.g., interdigitating).

Figure 7 illustrates the side view of an approximated array of registered or aligned addressable working and counterelectrodes and the complementary binding surface wherein conducting polymers are grown from the surfaces of the electrodes across the gap between the electrode array and the binding domains so as to extend the potential field around the ECL label of the sample to increase the efficiency of the ECL reaction.

Figure 8 illustrates the side view of an approximated array of registered or aligned addressable working and counterelectrodes and the complementary binding surface with

conducting particles interspersed between both components to extend the potential field. By extending the potential field around the ECL label of the sample the efficiency of the ECL reaction is enhanced. The conducting particles can be magnetic to permit ready manipulation.

Figure 9 illustrates the side view of an approximated array of registered or aligned addressable working and counterelectrodes and the complementary binding surface wherein the electrodes have fine projections extending into the gap between the electrode surface and the binding domains in order to extend the potential field around the ECL label of the sample, to increase the efficiency of the ECL reaction.

Figure 10 illustrates the side view of an approximated array of registered or aligned addressable working and counterelectrodes and the complementary binding surface where the surfaces are not parallel, but are instead conformed one to the other in a complementary fashion.

Figure 11 illustrates the side view of a support having a metallic layer thereon to provide a single electrode and binding surface assembly in the form of a cassette. An array of self-assembled monolayers ("SAMs") is patterned on the metallic layer.

Figure 12 illustrates the side view of a support having a metallic layer thereon to provide a single electrode and binding surface assembly in the form of a cassette. An array of SAMs is patterned on the metallic layer and conducting microparticles are shown interspersed among the patterned SAMs so as to extend the potential field around the ECL label of the sample, to increase the efficiency of the ECL reaction.

Figure 13 illustrates the side view of a support having a metallic layer thereon to provide a single electrode and binding surface assembly in the form of a cassette. An array of self assembled monolayers or SAMs is patterned on the metallic layer and the growth of a conducting polymer and/or fiber from the ECL label so as to extend the potential field around the ECL

label of the sample to increase the efficiency of the ECL reaction, is illustrated.

Figure 14 is a diagram of a support having an array of electrode pairs controlled by a computer.

Figure 15 is a diagram of a support having an array of electrode pairs.

Figure 16 is a diagram of a support having an array of electrode pairs and computer system for controlling the energization of each electrode pair.

Figure 17 is a diagram of a support having an array of electrode pairs and a computer system with a plurality of voltage sources and multiplexers for controlling the energization of each electrode pair.

Figure 18 is a diagram of a support having an array of electrode pairs and a computer system with a plurality of switched voltage sources for controlling the energization of each electrode pair.

Figures 19 (a)-(e) are plan views of several alternative electrode-counter-electrode pair combinations.

Figure 20 illustrates a support with a completed sandwich assay.

Figure 21 illustrates two opposing PMAMS surfaces on supports.

Figure 22A illustrates an array of microfluidics guides (2201) and a fibril mat (2200).

Figure 22B illustrates binding domains (2202).

Figure 23A illustrates an apparatus for forming a fibril mat by vacuum filtration.

Figure 23B illustrates a fibril mat (2304) on a filter membrane (2303).

Figure 24 illustrates the use of rollers to produce fibril mats.

Figure 25 shows a schematic of a multi-layer fibril mat, in which the upper layer has binding domains used for assays.

Figure 26 shows a schematic of a fibril derivatized with moieties that enhance non-specific binding, and several species, both biological and non-biological are bound to the surface.

Figure 27 shows a schematic of a fibril derivatized with moieties that enhance non-specific binding and several species bound to a derivatized fibril with some species additionally bound to ligands.

Figure 28 illustrates several species covalently attached to a fibril and some species are further bound to additional entities.

Figure 29 illustrates the use of a multilayer fibril mat as an optical filter that, depending on the position of a source of light on or within the mat, may allow light to pass and/or may absorb and/or scatter light.

Figure 30A illustrates cyclic voltammograms from electrochemical measurements on carbon fibril mat electrodes.

Figure 30B illustrates cyclic voltammograms from electrochemical measurements on gold foil electrodes.

Figure 31 compares an electrochemical property of fibril mats as a function of the thickness of the mat and the scan rate.

Figure 32 shows a plot that illustrates that non-specific binding on fibrils generally increases as the concentration of fibrils in a protein solution increases.

Figure 33 demonstrates that the use of surfactants can reduce non-specific binding between ECL-TAG1-labeled protein and carbon fibrils.

Figure 34 shows a schematic of a top view of an experimental cell used to measure electrochemical properties and ECL on a fibril mat electrode.

Figure 35 shows an ECL signal obtained using a fibril mat as an electrode and 1000 pM TAG1 (solid line) in solution and a signal from assay buffer (no TAG1) (dashed line).

Figure 36 shows a schematic of a two surface PMAMS device, in which two arrays of supported electrodes are separated by a patterned dielectric layer.

Figure 37 illustrates an apparatus with a plurality of binding domains (3702) on one support and an electrode and counterelectrode on another support.

Figure 38 shows a cassette where binding domains are presented on the surfaces of distinct objects supported on the counter electrode.

Figure 39 shows a gel in contact with a working and counterelectrode.

Figure 40 shows a graph of ECL intensity and a cyclic voltammogram from an ECL labeled gel in contact with a working and counterelectrode.

Figure 41 shows a graph of ECL intensity and a cyclic voltammogram from a non-ECL labeled gel in contact with a working and counterelectrode.

Figure 42 shows a schematic for a two-surface cassette used for ECL.

Figure 43 demonstrates that fibril mats can be used as electrodes for ECL of Antibody-TAG1 adsorbed to the mats.

Figure 44A shows ECL intensity of a TAG1 labeled protein immobilized on an electrode.

Figure 44B shows the cyclic voltammogram of a coated electrode.

Figure 45A shows quasi-reversible repetitive generation of ECL signal from an immobilized ECL TAG1 labeled protein.

Figure 45B shows the cyclic voltammogram of a coated electrode indicating partial preservation of the coating.

Figure 46A shows irreversible generation of ECL signal from an immobilized ECL TAG1 labeled protein.

Figure 46B shows the cyclic voltammogram of a coated electrode indicating substantial loss of the coating.

Figure 47 shows a multi-array ECL apparatus and a microprocessor containing controller means for generating and analyzing ECL signals.

5. DETAILED DESCRIPTION OF THE INVENTION

Accordingly, the invention includes in a broad aspect cassettes for conducting a plurality of electrochemiluminescence assays. The cassettes are formed of supports having thereon a plurality of binding domains able to specifically bind one or more analytes of interest. The binding domains are prepared as patterned, multi-array multi-specific surfaces ("PMAMS") on the support. The PMAMS offer a significant improvement from ECL assay methods previously known by, e.g., greatly increasing the density of assays that can be performed and allowing for a plurality of different assays that may be rapidly or simultaneously performed. The cassette may include a plurality of electrodes able to selectively trigger ECL emission of light from ECL labeled reagents bound to the binding domains. Fig. 47 shows a multiarray ECL apparatus having electrodes 4700, 4704, a matrix 4702 with binding domains 4706 and a microprocessor containing controller 4720 means for generating and analyzing an ECL signal connected via leads 4710-4716.

In the embodiment of the invention shown in Fig. 1, a cassette comprises two supports 10, 12 wherein a plurality of binding domains 14 are present on a surface of a first support 10 and a plurality of electrode/counterelectrode pairs 16 are present on a surface of second support 12. The binding domains and electrode/counterelectrode pairs are aligned so that each of the plurality of electrode/counterelectrode pairs 16 is placed adjacent to a different one of the plurality of binding domains 14 when the first and second supports 10, 12 are brought together. First support 10 underlying binding domains 14 is preferably a PMAMS with a gold film surface and transparent binding domains. Second support 12 is preferably a transparent flat plastic sheet having transparent electrode/counterelectrode pairs 16 thereon. Binding domains 14 are preferably prepared by

micro stamping an organic self assembled monolayer (composed of individual monomers) pattern on the support surface, where the monomer has a biotin or linking moiety. Avidin or streptavidin is then bound to the exposed biotin (see e.g., U.S. Patent 5,093,268). Binding reagents are then applied by applying a discrete amount of a suitable biotin-labeled binding reagent such as biotin labeled antibody, which is able to selectively bind to the analyte of interest, to the locations on the support surface where the monolayer had been stamped. Fig. 1A illustrates a system comprising a cassette (Fig. 1) contained in a housing (11).

In certain embodiments of the invention, it is desirable to reproducibly immobilize a specified or predetermined amount of one or more reagents on a surface. Immobilization broadly applies to any method by which a reagent is attached to a surface, including but not limited to: covalent chemical bonds; non-specific adsorption; drying a reagent on a surface; electrostatic interactions; hydrophobic and/or hydrophilic interactions; confinement or entrainment in liquids or gels; biospecific binding, (e.g., ligand/receptor interactions or hybridization of oligonucleotides); metal/ligand bonds; chelation, and/or entanglement in polymers.

The amount of reagent immobilized on a surface may be predetermined in several ways. For example, the amount of reagent on a surface may be specified by one or more volume and/or area elements in which the reagent is present. It may also be specified by the number of individual molecules of a reagent that are immobilized on a surface. The amount of reagent may be specified in terms of the density of a particular reagent in a given region. The amount of reagent may be specified as a percentage of a surface bearing a particular reagent, either with regard to the total area of the surface, or relative to the amounts of other reagents present on the surface. The amount of reagent may also be defined as the quantity of reagent that must be present on a particular surface to give sufficient ECL

intensity so as to make an assay achieve a desired specificity. In a specific example, a 1 cm² area of a gold surface may be coated with a monolayer of alkanethiols.

Reagents may also be reproducibly immobilized on coated surfaces. The coating may serve to enhance immobilization for some reagents and/or reduce or prohibit immobilization for other reagents. The surface may be completely coated or the surface may be partially coated (i.e. a patterned coating). The coating may be uniform in composition, or it may contain elements of different composition. In a specific example, the coating may be a patterned monolayer film that immobilizes immunoglobulin G via covalent chemical bonds in some areas, and prevents its immobilization in others.

The coating may also serve to predetermine the amount(s) of one or more reagents immobilized on the surface in subsequent steps or processes. Alternatively, the amount of a particular reagent may be controlled by limiting the amount of reagent that is deposited.

Having a surface that has reagents (or a coating) immobilized in a quantitative, reproducible fashion gives the ability to reproducibly and quantitatively measure an ECL signal from a sample, thus allowing calibration.

Preferably, electrode/counter-electrode pairs 16 are of sub-centimeter size and are fabricated along with electrode leads 20 (e.g., of a transparent metal film) by methods well known for the fabrication of liquid crystal displays and electrochromic display panels. Electrode leads 20 are connected by electrical connections 19 to a waveform generator means 18. Advantageously, the electrode/ counter-electrode pairs are individually addressable under computer control so that electrical potential can be selectively applied to discrete binding domains. A light detector means 22 and a digital computer means 24 are provided to record and analyze the results when ECL emissions have been stimulated from a suitable label bound to a binding domain.

Fig. 1A illustrates a system comprising a cassette, electrical leads, waveform generator, means of light detection and digital computer as described in Fig. 1 contained in a housing (11). The cassette is inserted into the housing through opening (15).

In another embodiment, one working electrode is used to simultaneously generate an ECL signal at a plurality of binding domains. In this embodiment, the ECL signal from each binding domain is identified through the use of light imaging equipment.

Broadly, the assays conducted using cassettes according to the invention are assays that benefit from the use of a plurality of discrete binding domains. For example, use of such cassettes allows rapid and/or concurrent detection or measurement of a wide variety of analytes of interest. In a preferred embodiment, the assays according to the invention are also those that benefit from the use of an ECL labeled reagent, analyte or binding surface. An ECL assay according to the invention comprises contacting a plurality of binding domains with a sample suspected of containing an analyte of interest and triggering an ECL emission from a bound ECL label, wherein the ECL label is on the analyte or a competitor of the analyte, on a reagent that binds to the analyte or on the plurality of binding domains.

The invention also provides ECL assay methods for detecting or measuring an analyte of interest, comprising (a) contacting one or more of a plurality of discrete binding domains, said plurality of binding domains being immobilized on a surface of one or more supports, in which said contacting is with a sample comprising molecules linked to an electrochemiluminescent label, wherein said sample does not contact any electrodes or counterelectrodes during said contacting step; (b) bringing an electrode into proximity to said one or more of a plurality of binding domains; (c) applying a voltage waveform effective to trigger ECL at said one or more of a plurality of binding domains; and detecting or measuring ECL.

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In another embodiment, the invention provides ECL assay methods for (a) contacting one or more of a plurality of discrete binding domains, said plurality of binding domains (i) being immobilized on a surface of one or more supports, and (ii) being spatially aligned with and in proximity to a plurality of electrode and counterelectrode pairs, in which said contacting is with a sample comprising molecules linked to an electrochemiluminescent label; (b) bringing an electrode and counterelectrode into proximity to said one or more of a plurality of binding domains; (c) applying a voltage waveform effective to trigger electrochemiluminescence at said one or more of a plurality of binding domains; and (d) detecting or measuring electrochemiluminescence.

The plurality of binding domains on the support are allowed to interact with samples to be assayed. PMAMS may be further contacted with solutions containing necessary reagents to complete an assay. The binding surface is then contacted (e.g., pressed) with a surface of a complementary electrode (preferably a clean, or virgin, electrode) which is then used to apply an electrical potential to stimulate ECL.

In a preferred method of conducting an assay using the apparatus of Fig. 1, a sample suspected of containing an analyte of interest is applied to plural binding domains 14 together with ECL labeled reagents that are suitable for detecting the analyte. Support 11 and support 12 are then brought together so that each of the plural binding domains 14 is located between the electrode and counterelectrode of a different one of plural electrode/counterelectrode pairs 16 and the sample is contained therebetween. It should be noted that the electrode and counterelectrode pair need not make mechanical contact with the binding domain in order to stimulate ECL when an appropriate potential is applied across the electrode and counterelectrode pair. An electrical potential waveform suitable for triggering an ECL emission is applied via electrical connection 19 from waveform generator means 18 to plural electrode/counterelectrode

pairs 16. Any signal emitted by an ECL label present on plural binding domains 14 is detected by light detection means 22 and recorded and analyzed by digital computer means 24.

The invention provides a method of detecting in a volume of a multicomponent, liquid sample a plurality of analytes of interest which may be present in the sample at various concentrations.

Broadly a plurality of analytes may be detected from a multicomponent sample in less than 10^{-3} molar concentrations. Preferably a plurality of analytes may be detected at less than 10^{-12} molar concentrations from a multicomponent sample.

The invention provides for detection from a multicomponent sample which may be performed as heterogeneous assays, i.e., assays in which a plurality of unbound labeled reagents are separated from a plurality of bound labeled reagents prior to exposure of the bound labeled reagents to electrochemical energy, and homogeneous assays, i.e., assays in which a plurality of unbound labeled reagents and bound labeled reagents are exposed to electrochemical energy together.

In the assays of the present invention, the electromagnetic radiation used to detect a particular analyte is distinguishable from the electromagnetic radiation corresponding to other analytes by identifying its position and/or location as one or more features of a pattern, said pattern corresponding to the pattern of the binding domains in the PMAMS.

In the homogeneous assays of the present invention, the electromagnetic radiation emitted by the bound labeled reagents either as an increase or as a decrease in the amount of electromagnetic radiation emitted by the bound labeled reagents in comparison to the unbound reagents, or by detection of electromagnetic radiation emitted from sources corresponding in space to one or more features of a pattern corresponding to the pattern of the binding domains in the PMAMS.

In a specific example of the method of the invention shown in Fig. 20, a sandwich assay is conducted on a support (5)

with a plurality of binding domains (BD) on its surface that are specific for binding a particular analyte (An). When a sample suspected of containing the analyte is applied to the binding domains, the analyte is bound to the binding domains. Antibodies (Ab), which are suitable for selectively binding analyte (An) and have been labeled with an ECL moiety (TAG) to form Ab-TAG, are then applied to the analyte on the binding domains. After excess, unbound Ab-TAG is washed off the binding domains, a potential waveform suitable for triggering electrochemiluminescence is applied to the TAG by electrodes (not shown) to trigger an ECL emission from any TAG on the binding domains. The ECL signal is detected by light detection means and recorded by digital computer means (e.g., as illustrated at 22 and 24 in Fig. 1).

Further embodiments, features and variations of the invention are provided as described hereinbelow.

5.1. PREPARATION OF A BINDING SURFACE

To better understand the invention, a more detailed description of the preparation of binding domains on a support is provided. A patterned array of binding domains on a surface that are specific for a plurality of analytes is referred to herein as a patterned, multi-array multi-specific surface or PMAMS. PMAMS are prepared on a support, for example, by patterning of self-assembled monolayers ("SAMs") (Ferguson et al, 1993, *Macromolecules* 26(22):5870-5875; Prime et al., 1991, *Science* 252:1164-1167; Laibinis et al., 1989, *Science* 245:845-847; Kumar et al., 1984, *Langmuir* 10(5):1498-1511; Bain et al., 1989, *Angew. Chem.* 101:522-528). Surface patterning methods also include the use of physical etching (e.g., micromachining) (Abbott et al., 1992, *Science* 257:1380-1382; Abbott, 1994, *Chem. Mater.* 6(5):596-602), microlithography (Laibinis et al., 1989, *Science* 245:845-847), attachment of chemical groups to the surface through the use of photoactivatable chemistries (Sundberg et al., 1995, *J. Am. Chem. Soc.* 117(49):12050-12057), and micro-stamping techniques (Kumar et al., 1994, *Langmuir* 10(5):1498-1511; Kumar

et al., 1993, Appl. Phys. Lett. 63(14):2002-2004). Other surface patterning methods include procedures for the spatially controlled dispensing of fluids or particles (e.g., micropen deposition (e.g., using a microfluidic guide to deliver onto a surface using X-Y translation)), microcapillary filling (Kim et al., 1995, Nature 376:581), Ink-Jet technology, or syringe dispensers. Combinations of these techniques may be used to provide complex surface patterns. In Fig. 5A, a support 600 is shown with shape independent binding domains that are represented, simply for illustration purposes, as geometric shapes 602 to indicate that different binding specificities may be present on a single support. Surface 604 between binding domains may be alternatively hydrophobic or hydrophilic to confine deposition of binding reagent to form binding domains. Binding domains and/or the surface(s) between binding domains may be alternatively prone and resistant to nonspecific binding, and/or they may be prone and resistant to the attachment of binding reagents via covalent or non-covalent interactions. In the case where non-specific binding through hydrophobic interactions is not the desired method for attachment of binding chemistries to the surface, detergent may be added to prevent incidental non-specific binding from occurring.

The binding domains are broadly from 0.1 μm to 1 mm in width or diameter or widest dimension depending upon the geometry of the domain). The surfaces are selectively derivatized to have specific binding components exposed to e.g., the ECL assay solution. Additionally, non-specific interactions at the binding domains are decreased while maintaining a specific binding moiety by incorporating moieties such as polyethyleneglycols on the exposed surface of the discrete binding domains (Prime et al., 1993, J. Chem Soc. 115:10714-10721; Prime et al., 1991 Science 252:1164-1167; Pale-Grosdemange et al., 1991, J. Am. Chem. Soc. 113:12-20).

The PMAMS may contain broadly from 2 to 10^8 binding domains. Preferably, the number of binding domains is from 50 to

500. In still other embodiments, the number of binding domains is from 25 to 100.

The support may be a variety of materials including but not limited to glass, plastic, ceramic, polymeric materials, elastomeric materials, metals, alloys, composite foils, semiconductors, insulators, silicon and/or layered materials, etc. Derivatized elastomeric supports can be prepared, e.g., as described by Ferguson et al., 1993, *Macromolecules* 26:5870-5875; Ferguson et al., 1991, *Science* 253:776-778; Chaudhury et al., 1992, *Science* 255:1230-1232.

The surface of the support on which PMAMS are prepared may contain various materials, e.g., meshes, felts, fibrous materials, gels, solids (e.g., formed of metals) elastomers, etc. The support surface may have a variety of structural, chemical and/or optical properties. For example, the surface may be rigid or flexible, flat or deformed, transparent, translucent, partially or fully reflective or opaque and may have composite properties, regions with different properties, and may be a composite of more than one material. The surface may have patterned surface binding regions and/or patterned regions where catalyses may occur according to the invention on one or more surfaces, and/or an addressable array of electrodes on one or more surfaces. The surfaces of the supports may be configured in any suitable shapes including planar, spheroidal, cuboidal, and cylindrical. In a specific embodiment, the support bearing a PMAMS is a dipstick. In another embodiment the support bearing a PMAMS contains carbon, e.g., graphite, glassy carbon or carbon black.

In one embodiment, a support bearing a PMAMS contains one or more carbon fibers. These fibers may be amorphous or graphitic carbon. They may also be carbon nanotubes, buckeytubes or members of the fullerene family.

In a preferred embodiment, a support bearing a PMAMS contains one or more carbon fibrils (Hyperion FibrilsTM) (U.S. Patent No. 4,663,230). Individual carbon fibrils (as disclosed

in U.S. Patent No.'s 4,663,230, 5,165,909, and 5,171, 560) may have diameters that range from about 3.5 nm to 70 nm, and length greater than 10^2 times the diameter, an outer region of multiple essentially continuous layers of ordered carbon atoms and a distinct inner core region. Simply for illustrative purposes, a typical diameter for a carbon fibril may be approximately between about 7 and 25 nm, and a typical range of lengths may be 1 μm to 10 μm .

Carbon materials can be made to form aggregates. As disclosed in U.S. Patent No. 5,110,693 and references therein, two or more individual carbon fibrils may form microscopic aggregates of entangled fibrils. These aggregates can have dimensions ranging from 5 nm to several cm. Simply for illustrative purposes, one type of microscopic aggregate ("cotton candy or CC") resembles a spindle or rod of entangled fibers with a diameter that may range from 5 nm to 20 μm with a length that may range from 0.1 μm to 1000 μm . Again for illustrative purposes, another type of microscopic aggregate of fibrils ("birds nest, or BN") can be roughly spherical with a diameter that may range from 0.1 μm to 1000 μm . Larger aggregates of each type (CC and/or BN) or mixtures of each can be formed (*vide infra*).

Fibrils that can be used in a support include but are not limited to individual fibrils, aggregates of one or more fibrils, suspensions of one or more fibrils, dispersions of fibrils, mixtures of fibrils with other materials (e.g., oils, paraffins, waxes, polymers, gels, plastics, adhesives, epoxies, teflon, metals, organic liquids, organic solids, inorganic solid, acids, bases, ceramics, glasses, rubbers, elastomers, biological molecules and media, etc.) as well as combinations thereof.

The fibrils may be magnetic in some cases and non-magnetic in others. The extent to which fibrils can be made magnetic or non-magnetic is controlled by the amount of catalyst that is in the fibril as a result of the fibril production process, such process being disclosed in U.S. Patents 4,663,230,

5,165,909, and 5,171,560. PMAMS are located on, in, or in proximity to the supports described *supra*.

PMAMS can be generated from different types of surface binding groups. Self-assembling monolayers that can be used to form a monolayer on a surface to which they bind, include but are not limited to alkane thiols (which bind gold and other metals), alkyltrichlorosilane (e.g., which bind silicone/silicone dioxide), alkane carboxylic acids (e.g., which bind aluminum oxides) as well as combinations thereof. The monolayer may be formed first and then linking chemistry used to attach binding reagents. Derivatization after self-assembly produces a more perfect two-dimensional crystalline packing of the monolayer on a support surface with fewer pin holes or defects. The monolayer can be derivatized with the binding reagents before or after self-assembly. Regular defects in the monolayer may be desirable, and can be obtained by derivatization prior to self-assembly of the monolayer or the support surface. If the derivatized group (e.g., exposed binding group) on the binding reagent is sterically large, it may create a close-packed surface at the exposed end, but with regular gaps at the metal surface. This is useful for allowing charge to flow through these regular gaps to the ECL labeled moieties bound to the portion contacting the sample solution.

The preparation of incomplete monolayers is known in the art. Other procedures for the preparation of incomplete monolayers include but are not limited to: the formation of monolayers from dilute solutions of binding reagent, the termination of the monolayer forming reaction before completion, the damaging of more complete monolayers with radiation (e.g., ionic particles), light or chemical reagents. In one embodiment, repeated stamping without re-inking the stamp can give a range of defective monolayers (Wilbur et al., 1995, Langmuir, 11:825).

PMAMS can be generated on the surface of matrices. Matrices may be highly conducting, e.g., metal electrodes or conducting polymer films; or matrices may be insulators; or

matrices semi-conducting and/or of medium conductivity. The matrix material may be an ionic conductor or a porous material. Such porous materials may be utilized as support material and/or a conductive material and/or a filter material and/or a channelling material (e.g., allowing passage of fluids, ionic species etc.).

The porous material may be combined with additional materials. For example, composite structures may be fabricated of porous materials with additional porous materials, conductive materials, semiconductive materials, channelling structures and/or solutions (e.g., ionic fluids). Such composites may be laminar structures, sandwich structures, and/or interspersed composites. A solid matrix may be used which is a porous material supported on a metal electrode. Alternatively, a porous material is sandwiched between conducting materials, semiconducting materials or a combination of semiconducting and conducting materials. One or more binding domains may be contained on one continuous slab of the porous material and/or may be located on a plurality of discrete objects on the support each with one or more binding domains. The porous material (e.g., gel) surface may be flat, hemispherical or take on any regular or irregular shape and/or may have a variety of physical properties (e.g., elastomeric, rigid, low density, high density, gradient of densities, dry, wet etc.) and/or optical properties (e.g., transparent, translucent, opaque, reflective, refractive etc.) and or electrical properties (e.g. conductive, semiconductive, insulating, variably conductive, for example wet vs. dry etc.).

A pattern of channels may be formed in the matrix. The porous material layers may be from 5 microns to 2000 microns thick. The porous material layers may also be thicker than 2 mm.

The pores may extend partially and/or fully through the material or may be part of a network of pores. These pores may have dimensions ranging broadly from 50 Å to

10000 μm . In a preferred embodiment, the material has some pores with dimensions ranging from 200 Å to 500 Å and some pores with dimensions ranging from 0.5 μm to 100 μm .

The porosity of the material may be constant throughout the material or may increase or decrease as a function of the position in the material. The material may have a wide variety of pores of different size distributed in a disorganized and/or random manner.

The porous material may be a composite of more than one materials.

For example, the material may have some pores that are large enough to pass objects as large as biological cells, some pores that can pass biological media as large as proteins or antibodies, some pores that can pass only small (< 1000 molecular weight) organic molecules, and/or combinations thereof.

The porosity of the material may be such that one or more molecules, liquids, solids, emulsions, suspensions, gases, gels and/or dispersions can diffuse into, within and/or through the material. The porosity of the material is such that biological media can diffuse (actively or passively) or be forced by some means into, within and/or through the material. Examples of biological media include but are not limited to whole blood, fractionated blood, plasma, serum, urine, solutions of proteins, antibodies or fragments thereof, cells, subcellular particles, viruses, nucleic acids, antigens, lipoproteins, liposaccharides, lipids, glycoproteins, carbohydrates, peptides, hormones or pharmacological agents. The porous material may have one or more layers of different porosity such that biological media may pass through one or more layers, but not through other layers.

The porous material may be able to support a current due to the flow of ionic species. In a further refinement, the porous material is a porous water-swollen gel, for example polyacrylamide or agar. A variety of other gel compositions are available (for example see Soane, D. S. *Polymer Applications for Biotechnology*; Soane, D. S., Ed.; Simon & Schuster: Englewood

Cliffs, NJ, 1992 or *Hydrogels in Medicine and Pharmacy*, Vol. I-III; Peppas, N.A. Ed.; CRC Press: Boca Raton, FL, 1987).

Binding domains can be attached to matrices by covalent and non-covalent linkages. (Many reviews and books on this subject have been written; some examples are Tampion J. and Tampion M.D. *Immobilized Cells: Principles and Applications* Cambridge University Press: NY, 1987; *Solid Phase Biochemistry: Analytical and Synthetic Aspects* Scouten, W.H. Ed., John Wiley and Sons: NY, 1983; *Methods in Enzymology, Immobilized Enzymes and Cells*, Pt. B Mosbach, K. Ed., Elsevier Applied Science: London, 1988; *Methods in Enzymology, Immobilized Enzymes and Cells*, Pt. C Mosbach, K. Ed., Elsevier Applied Science: London, 1987; *Methods in Enzymology, Immobilized Enzymes and Cells*, Pt. C Mosbach, K. Ed., Elsevier Applied Science: London, 1987; see also *Hydrogels in Medicine and Pharmacy*, supra). For example, a protein can be attached to a cross linked copolymer of polyacrylamide and N-acryloylsuccinimide by treatment with a solution of the protein. The binding domains may also be integrated into a porous matrix in a step prior to polymerization or gelation. In one embodiment, binding domains may be attached to uncrosslinked polymers by using a variety of coupling chemistries. The polymers may then be crosslinked (for example using chemistries which include amide bonds, disulfides, nucleophilic attack on epoxides, etc.) (see for example: Pollack et. al., 1980, J. Am. Chem. Soc. 102(20):6324-36). Binding domains may be attached to monomeric species which are then incorporated into a polymer chain during polymerization (see Adalsteinsson, O., 1979, J. Mol. Catal. 6(3): 199-225). In yet another embodiment, binding domains may be incorporated into gels by trapping of the binding domains in pores during polymerization/gelation or by permeation of the binding domains into the porous matrix and/or film. Additionally, binding domains may be adsorbed onto the surface of porous matrices (e.g., polymer gels and films) by nonspecific adsorption caused for example by hydrophobic and/or ionic interactions. Biotin may

be advantageously used as a linking or binding agent. Avidin, streptavidin or other biotin binding agents may be incorporated into binding domains.

PMAMS can be generated on porous materials (e.g., gels) with varying pore size and solvent content. For example, polyacrylamide gels varying in pore size can be made by varying the concentration of acrylamide and the degree of crosslinking.

On such PMAMS with pore sizes smaller than the analyte, binding reactions will occur substantially on the surface of the gel. In this case, filtration and/or electrophoresis through the gel can be used to concentrate analytes at the surface of the gel and modulate the kinetics (e.g., increase the rate) of the binding reaction. Faster kinetics is advantageous in rapid assays (e.g., short times to results) and may generate increased sensitivity in a shorter time period.

On PMAMS with pore sizes larger than the analyte, binding reactions can occur on the surface as well as in the bulk of the gel. In this case, filtration can be used and/or electrophoresis can be used to increase the kinetics of binding and remove unbound species from the surface.

PMAMS formed on gels can be stored wet and/or they may be stored in a dried state and reconstituted during the assay. The reagents necessary for ECL assays can be incorporated in the gel before storage (by permeation into the gel or by incorporation during formation of the gel) and/or they can be added during the assay.

Patterned binding domains of a PMAMS can be generated by application of drops or microdrops containing each binding domain in the matrix in a liquid form to a substrate. Solidification and/or gelling of the liquid can then be caused by a variety of well known techniques (polymerization, crosslinking, cooling below the gelling transition, heat). Agents that cause solidification or gelation may be included in the drops, so that at some time after dispensing, the drops solidify and/or gel. A subsequent treatment (e.g., exposure to light, radiation and/or

redox potential) may be used to cause solidification and/or gelation. In other embodiments such drops or microdrops may be slurries, pre-polymeric mixtures, particulate groups, and/or substantially solid drops. Additionally vapor phase deposition may be utilized.

Patterning can also be achieved by forming a layered structure of matrices each containing one or more binding domains. For example, agarose linked (by standard chemistries) to an antibody could be poured into a container and allowed to gel by cooling. Subsequent layers containing other antibodies could then be subsequently poured on the first layer and allowed to gel. The cross section of this layered structure gives a continuous surface presenting a plurality of distinct binding domains. Such cross sections may be stacked and another cross section may be cut to create a PMAMS surface with even greater density of binding domains. Alternatively, lines of a matrix containing a given binding element are laid down adjacent to one another and/or stacked. Such structures may also be cut in cross-section and utilized as a PMAMS surface.

Patterning can also be achieved by taking advantage of the ability of some matrices to achieve separation. For example, a mixture of nucleic acid probes could be separated by electrophoresis in a polyacrylamide slab generating a surface presenting a plurality of distinct binding domains.

Microfluidics guides may also be used to prepare the PMAMS binding domains on a support. A partial list of microfluidic guides includes hollow capillaries, capillaries made of and/or filled with a matrix (e.g., a porous or solvent swollen medium), solid supports which can support a thin film or drop of liquid. The capillary may be solid and reagents flow along the outside surface of the capillary, a reagent fluid reservoir may be exposed to a porous matrix tip which is brought into contact with a PMAMS surface. For example, the reagent reservoir may be continuously or periodically refilled so that a given porous matrix tip may reproducibly deposit reagents (e.g., alkane thiols

to form monolayers and/or binding reagents etc.) a plurality of times. Additionally, varying the porosity of the tip can be utilized to control reagent flow to the surface. Different or identical binding reagents may be present in a plurality of capillaries and/or multiple distinct binding agents may be present in a given capillary. The capillaries are brought into contact with the PMAMS (e.g., patterned SAM) surface so that certain regions are exposed to the binding reagents so as to create discrete binding domains. Different binding reagents, each present in a different microfluidic guide are delivered concurrently from the fluidic guide array onto a metal surface, SAM, etc, as desired. Microfluidic guides can also be used to ink a microstamp with a desired molecule prior to application to the support surface. For example, individual microfluidic guides can be used to apply different binding reagents linked to a moiety that promotes adsorption to the surface of the support (e.g., a free thiol on a hydrocarbon linker, which promotes adsorption to gold), to form a PMAMS. Thus, for example, a microstamp inked via the use of microfluidic guides with antibodies of different specificities that have incorporated a linker with a free thiol, can be used to apply such antibodies in desired areas on a gold surface to form discrete binding domains of a PMAMS.

Another approach to patterned fluid delivery involves the use of microprinting devices which deliver microdrops of fluid by ejection of the drop through a small orifice (e.g., an Ink-Jet printer). The ejection drops in these devices may be caused by different mechanisms including heating, electrostatic charge, and/or pressure from a piezo device. Patterning of more than one liquid can be achieved through the use of multiple orifices and/or one orifice and appropriate valving.

In one method for preparation of a PMAMS, microfluidic guides are used to deliver (preferably concurrently) directly onto discrete regions on a surface, drops containing the desired binding reagents, to form discrete binding domains. The binding

reagents may contain a functional chemical group that forms a bond with a chemical group on the surface to which it is applied. In another variation, binding reagents in the drop are nonspecifically adsorbed or bound to the surface (e.g., dried on the surface).

Alternatively, drop(s) deposited on a surface contain reagents that can form a matrix. This matrix may be a solid, polymer or a gel. The formation of the matrix may be by evaporation of solvent. It may be by polymerization of monomeric species. It may be by cross-linking of preformed polymers. It may be by modulating temperature (e.g., cooling and/or heating). It may be by other methods. For example, a polymeric species may be cooled through a cooling transition or by addition of a reagent that causes gelling. The formation of the solid matrix may be induced by generation of reactive species at an electrode (including the substrate), by light (or other radiation) by addition of reagents that induce solidification or gelling, by cooling or heating. Additionally, the surface may contain catalysts capable of initiating matrix formation (e.g. gelling or polymerization).

In a preferred technique, patterned hydrophilic/hydrophobic regions to prevent spreading of applied fluids or gels can be used. Such a fluid or gel may contain binding reagents to be linked to a surface on a support to form a binding domain of the PMAMS. In this case, use of such a hydrophilic/hydrophobic border aids in confining the produced binding domain to a discrete area. Alternatively, the fluid contains reagents which can form a matrix on the surface and binding reagents are contained within a defined region when deposited on a surface. For example, hydrophilic/hydrophobic border aids may be utilized to confine the drop to a defined region. Additionally, either the hydrophilic or hydrophobic areas may present groups which can be incorporated (e.g., covalently or non-covalently bound) into the matrix, allowing for a more stable adhesion of the matrix to the substrate (Itaya and

the fluid or gel that is applied is the sample containing the analyte of interest, and the sample is applied to a prepared PMAMS. In one preferred example, capillaries containing hydrophilic solutions can be used to deposit a solution onto discrete areas, creating hydrophilic domains surrounded by hydrophobic regions. Alternatively, hydrophobic binding domains surrounded by hydrophilic regions can be used with a hydrophobic fluid containing binding reagents or analyte(s)). Hydrophobic and hydrophilic are relative terms, with respect to each other and/or with respect to the sample to be applied, i.e., such that the spread or wetting of a fluid or gel sample applied to the binding domains is controlled. Further, controlled solution deposition from the microfluidics array may be accomplished using physical surface features (e.g., wells or channels on the surface). A microfluidics guide can be included in a cassette, or more preferably, used to apply specific reagents to a support prior to use.

More than one linking chemistry may be applied to the same support surface and/or a surface with both hydrophilic and hydrophobic binding domains can be created using multiple stamps. For example, an area where a hydrophilic binding domain is desired at position 1 and a hydrophobic binding domain is desired at position 2 can be prepared as follows. A first hydrophilic stamp is made which has a disk at position 1 and a larger ring at position 2. A second hydrophobic stamp is made with a disk at position 2 which fits inside the ring monolayer left by stamp 1. Finally, the surface is washed with a hydrophobic solution of monolayer components.

In particular, a PMAMS is generated by micro-contact printing, i.e., stamping. The monolayer so applied is composed of a surface-binding group, e.g., for a gold surface, a thiol group with an alkane (e.g., $(CH_2)_n$) spacer is preferred. A spacer group is linked (preferably covalently bound) to a linking group A. "A" can be, e.g., avidin, streptavidin or biotin or any other suitable binding reagent with an available complementary

binding partner "B". The A:B linkage may be covalent or non-covalent and some linkage chemistries known to the art that can be used are disclosed by, e.g., Bard et al. (U.S. Patent Nos. 5,221,605 and 5,310,687). "B" is further linked to a binding reagent such as an antibody, antigen, nucleic acid, pharmaceutical or other suitable substance for forming a binding domain that can bind to one or more analytes of interest in a sample to be tested. B may also be linked to an ECL TAG or label. Linking group B may be delivered to the SAM by means of a capillary or microfluidics guide array (Figs. 5A-5C) able to place a plurality of "B" reagents with different binding surface specificities on the monolayer "A" linkage. A and B can also be linked before or prior to being attached to the monolayer. As discussed, in Fig. 5A, shape independent binding domains are represented, simply for illustration purposes as geometric shapes 602 to indicate that different binding specificities may be present on a single support 600. Fig. 5B provides a top view of a microfluidic guide (e.g., capillary) array 606. The dots 610 are the guides in cross section. Fig. 5C provides a side view of a microfluidic guide array 608. The lines emerging from the top and bottom are individual microfluidic guides 610. The geometric shapes 612 on the lower aspect represent specific binding domains formed upon delivery of binding reagent from each individual capillary.

By way of example, after the first stamping discussed *supra*, the bare surface (e.g., gold) regions may be reacted with a second alkane thiol which does not have linking chemistry A and is of the opposite hydrophobicity/hydrophilicity of the first monolayer above. In this way, specific linking domains are prepared on a surface.

A binding reagent that is specific or for one analyte of interest may be used for each binding domain or a binding reagent may be used that specifically binds to multiple analytes of interest.

In yet another variation, a support surface may be stamped multiple times by materials (e.g., binding reagents, ECL labels, SAMs) having different linking chemistries and/or binding moieties as shown by Fig. 5A above.

The binding reagents that are patterned can be stable and/or robust chemical groups (e.g., that survive the conditions to which they are subjected) which are later linked to less stable or robust binding groups. Multiple binding linkages may be utilized so as to optimize the conditions of each step in the preparation of a PMAMS surface and/or simplify the manufacturing of PMAMS surfaces. For example, a first PMAMS surface may be fabricated in a generic fashion and then modified to create different PMAMS surfaces. In another example, a generic PMAMS surface may be reacted with a solution mixture of binding reagents which themselves contain binding domains which direct them to particular regions (e.g., binding domains) on the PMAMS surface. For example, a pattern of binding domains each presenting a different oligo(nucleotide) sequence is linked to the surface. This surface is then treated with a solution containing a mixture of secondary binding reagents, each linked to a oligo (nucleotide) sequence complementary to a sequence on the surface. In this way, patterning of these secondary binding elements can be achieved. Preferably, the oligo (nucleotide) sequences are 6-30mers of DNA. Certain sets of 6-30mer sequences may contain substantially similar sequence complementarity so that the approximate binding constants for hybridization are similar within a given set and discernably different from less complementary sequences. In another embodiment, the secondary binding elements are proteins (for example, antibodies).

Methods described to inhibit wetting or spread of applied reagents or sample on a surface as described in Section 5.13 *infra*, can also be used in the preparation of PMAMS (and/or in sample application). Applied potential (e.g., from the electrode/counterelectrode pair) may be used to further control

the deposition and/or spreading of reagents and/or samples (see, e.g., Abbott et al., 1994, Langmuir 10(5):1493-1497).

The PMAMS binding reagents may be located on materials that contain carbon. They may also be located on individual carbon fibrils or the PMAMS binding reagents may be located on aggregates of one or more fibrils. In many embodiments, the PMAMS binding reagents may be located on suspensions of one or more fibrils, dispersions of fibrils, mixtures of fibrils with other materials (described by way of example above) as well as combinations thereof.

The PMAMS binding reagents may be located on a plurality of individual fibrils and/or aggregates of fibrils localized on or in or in proximity to a support. In one example, the binding reagents are localized on dispersed individual fibrils or fibril aggregates. These fibrils or aggregates of fibrils may be localized spatially into distinct domains on a support, and may constitute binding domains as defined in this application.

In another example, individual such binding domains or a plurality of such binding domains are located in spatially distinct regions of the support. By way of a non-limiting example, individual such binding domains or collections of binding domains may be located in depressions, pits and/or holes in the support. In still another example, individual binding domains or a plurality of domains may be located in drops of water, gels, elastomers, plastics, oils, etc. that are localized on the surface of the support. In yet another example, individual binding domains may be localized on the support by a coating (which may be patterned) that has different binding affinities for different binding reagents and/or binding reagent/fibril ensembles.

Binding domains are desirably located on a plurality of individual fibrils and/or aggregates of fibrils may be prepared on a support by means of one or more microfluidic guides (e.g., a capillary). Different or identical binding reagents may be

present in or on a plurality of microfluidic guides and/or multiple distinct binding agents may be present in or on a given microfluidic guide. The capillaries may be brought into contact with the support (spotting) and/or may deliver the reagents while either the microfluidic guide and/or the surface is being scanned or translated relative to the other (i.e., a penlike method of writing). The microfluidic guide may deliver the binding reagents located on the fibrils to the support so that certain regions of the support are exposed to the fibril-binding reagent complex(es) so as to create a discrete binding domain(s). In a preferred aspect, different binding reagents, each present in a different microfluidic guide are delivered concurrently from the guide array onto the support. In one example, binding reagents and/or the fibrils on which they are localized are derivatized with a chemical functional group that forms a bond (e.g., covalent or non-covalent interaction) to the surface of the support. In some embodiments, the binding reagents and fibrils are non-specifically bound or adsorbed to the surface. In yet another aspect, the binding reagents localized on the fibrils may be delivered to depressions, pits and/or holes in the surface of the support. In another example, the binding reagents are delivered to a surface that is coated with a material that has a stronger or weaker binding affinity for certain binding reagents or binding reagent/fibril ensembles and so creates domains of the reagents that are localized spatially and distinctly from other binding reagents

The binding reagents are localized on one or more individual fibrils or aggregates of fibrils that are magnetic. In such a case, a magnetic support may attract the binding reagents localized on magnetic fibrils to the support.

The support may contain several distinct regions that are magnetic and are surrounded by regions that are not magnetic. Binding reagents localized on magnetic fibrils may be localized on magnetic regions of the support. In one example, the support may contain one or more distinct regions that are magnetic and

are surrounded by regions that are not magnetic, and the strength of the magnetic field in the magnetic regions can be modulated or switched. In this aspect, use of such a modulated or switchable magnetic field aids in affixing or releasing the binding reagents localized on fibrils from the surface of the support and so may serve to stir or mix said domains.

There are broadly from 2 to 10^8 binding domains and preferably from 25 to 500 domains.

The binding domains may be located on the working electrode and/or the counter electrode.

The different embodiments described herein for different types of PMAMS, supports, and electrodes and configurations thereof may also be practiced in combination with each other.

The PMAMS supports may be preserved (e.g., through protective surface coatings, drying the surface, robust packaging under vacuum or inert atmosphere, refrigeration and related methods) for later use.

5.2. BINDING REAGENTS

The binding domains of the invention are prepared so as to contain binding reagents that specifically bind to at least one analyte (ligand) of interest. Binding reagents in discrete binding domains are selected so that the binding domains have the desired binding specificity. Binding reagents may be selected from among any molecules known in the art to be capable of, or putatively capable of, specifically binding an analyte of interest. The analyte of interest may be selected from among those described in Section 5.10 *infra*, "ECL Assays That May Be Conducted." Thus, the binding reagents include but are not limited to receptors, ligands for receptors, antibodies or binding portions thereof (e.g., Fab, (Fab)₂), proteins or fragments thereof, nucleic acids, oligonucleotides, glycoproteins, polysaccharides, antigens, epitopes, cells and cellular components, subcellular particles, carbohydrate moieties, enzymes, enzyme substrates, lectins, protein A, protein

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G, organic compounds, organometallic compounds, viruses, prions, viroids, lipids, fatty acids, lipopolysaccharides, peptides, cellular metabolites, hormones, pharmacological agents, tranquilizers, barbiturates, alkaloids, steroids, vitamins, amino acids, sugars, nonbiological polymers, biotin, avidin, streptavidin, organic linking compounds such as polymer resins, lipoproteins, cytokines, lymphokines, hormones, synthetic polymers, organic and inorganic molecules, etc. Nucleic acids and oligonucleotides can refer to DNA, RNA and/or oligonucleotide analogues including but not limited to: oligonucleotides containing modified bases or modified sugars, oligonucleotides containing backbone chemistries other than phosphodiester linkages (see, for example, Nielsen, P.E. (1995) Annu Rev. Biophys. Biomol. Struct. 24 167-183), and/or oligonucleotides, that have been synthesized or modified to present chemical groups that can be used to form attachments to (covalent or non-covalent) to other molecules (where we define a nucleic acid or oligo(nucleotide) as containing two or more nucleic acid bases and/or derivatives of nucleic acid bases).

The PMAMS of the invention may have a plurality of discrete binding domains that comprises at least one binding domain that contains binding reagents that are identical to each other and that differ in specificity from the binding reagents contained within other binding domains, to provide for binding of different analytes of interest by different binding domains. By way of example, such a PMAMS comprises a binding domain containing antibody to thyroid stimulating hormone (TSH), a binding domain containing an oligonucleotide that hybridizes to hepatitis C virus (HCV), a binding domain containing an oligonucleotide that hybridizes to HIV, a binding domain containing an antibody to an HIV protein or glycoprotein, a binding domain that contains antibody to prostate specific antigen (PSA), and a binding domain that contains antibody to hepatitis B virus (HBV), or any subset of the foregoing.

antigens), different binding reagents that bind to different binding moieties will recognize the same analyte. As another example, antibodies to different cell surface antigens on a single cell will recognize the same cell. As yet another example, antibodies to different epitopes of a single antigen can be used as binding reagents to recognize the antigen.

In still a further embodiment, only binding reagent(s) that specifically bind a single analyte of interest are present in one or more binding domains. Alternatively, binding reagents that specifically bind more than one analyte of interest are present in one or more binding domains (e.g., a cross-reactive antibody). In a particular design, binding reagents can be used that bind a class of analytes, e.g., with similar characteristics.

Binding domains may also be incorporated into a PMAMS that contain binding reagents that are specific for a desired standard analyte and that are utilized as an internal standard (e.g., a binding domain which can be contacted with a sample containing a defined quantity of an analyte to which the binding reagents bind). Multiple binding domains containing binding reagents specific for the same analyte(s) can also be incorporated into a PMAMS so as to allow statistical averaging of analytical results. The binding reagents may not only be specific for the same analyte, but may be identical, thus recognizing the same binding moiety on the analyte. Thus, a plurality of binding domains (e.g., within a range of 2 to 10^8) can be prepared that specifically bind to the same binding moiety, so that the ECL readings can be statistically averaged to control for variation and improve accuracy. The plurality of binding domains on a PMAMS may be specific for a control analyte or an analyte of interest, or both, on a single support.

As another example, one or more discrete binding domains may be prepared with a known initial concentration number of ECL labels. The built-in ECL layer serves as a control to monitor, e.g., label degradation and temperature effects.

A binding reagent may be used that is an enzyme specific for a substrate (said substrate being the analyte of interest), in which a product of the enzymatic reaction upon the substrate is a reporter agent (an agent that is detectable), e.g., a product that triggers an ECL reaction, a fluorescent molecule, a substance that changes color upon contact with appropriate enzyme (e.g., a chromogenic substrate for horseradish peroxidase), etc. In an example of such an embodiment, the enzyme used as a binding reagent is glucose dehydrogenase (GDH), which can be used to detect or measure glucose in a sample. An ECL label is situated within or near to the binding domain containing the GDH. NADH is produced by the action of the enzyme upon glucose, NADH being capable of reacting with the ECL labels to promote ECL (Martin et al., 1993, *Anal. Chim. Acta* 281:475).

Binding domains containing binding reagents which increase background binding (i.e., that bind to a binding moiety present on the analyte of interest as well as on other analytes in the sample) can be used to increase signal to noise ratios during the detection or measurement of electrochemiluminescence. By way of example, where the analyte of interest is a specific cellular subpopulation (e.g., CD4⁺ cells) and the sample is a fluid sample (e.g., blood) that contains cells from a patient, antibody to sialic acid can be used as a binding reagent to increase background binding to virtually all cells in the sample (since sialic acid is a component of virtually all cell surface glycoproteins), and an antibody to a cell surface antigen specific to the cellular subpopulation (e.g., antibody to CD4) can then be used as a binding reagent (in the same or different binding domain as that containing the antibody to sialic acid).

5.3. VOLTAGE WAVEFORM

The voltage waveform (change in electrical potential/time) impressed upon the plurality of electrode and counterelectrode pairs of the ECL cell must be sufficient to trigger an ECL reaction. This voltage waveform usually is in the form of a uniform voltage sweep starting at a first voltage,

moving steadily to a second voltage, moving back through the first voltage to a third voltage and then back again to the first voltage. For example, the waveform may start at a first voltage in a range from -0.5 through 0.5 volts, up to a second voltage in a range from 1 through 2.5 volts and moving back through the first voltage to a third voltage ranging from 0.0 to -1 volts. As another example, in simpler waves, the voltage can be modified from 0.0 to +3.5 to 0.0. The voltage waveforms may incorporate linear ramps, step functions, and/or other functions. The voltage waveforms may incorporate periods of time when the voltage remains fixed at one potential. The applied potential may be controlled relative to one or more reference electrodes, or, no reference electrodes may be used. Additionally, negative potential may be used. Thus, the voltages used to induce ECL emissions from the cassette of the present invention will be readily selected for optimal ECL signal intensity and specificity for the ECL label and assay medium.

In some applications, the voltage is preferably varied as the light emitted from the binding domain is measured. This is particularly important to determine the threshold value of the electrical field necessary to cause the binding domain to emit light. In this case, the electrical potential applied at the binding domain starts at a value believed to be below the threshold required to emit light, and a first measurement is made of the light emitted. If no light is measured, or the light is below a predetermined threshold, the electrical potential applied across the electrode pair is increased under computer control, such as by a computer controlled voltage source and another light measurement is made. This process can be repeated until the predetermined appropriate amount of light is received. In this way, the voltage applied may be used as the assay signal.

The ECL signal may be generated from an AC voltage applied to the electrode pairs.

The ordinary artisan who is familiar with the voltage and current settings as disclosed, for example, by U.S. Patent

Nos. 5,324,457 and 5,068,088 will readily be able to select the optimum operating voltages and voltage sweep for triggering ECL emission.

The potential required for generating ECL may be generated by illumination of the working electrode surface if the working electrode is a semiconductor or contains another moiety that generates electrical current in response to light.

5.4. ADDRESSABLE ELECTRODES AND METHODS FOR USING THE SAME

Numerous methods may be used for addressing the plurality of electrode/counterelectrode pairs. Several illustrative such techniques are illustrated in Figs. 14-18. Shown in those figures by way of example are four electrode/counterelectrode pairs 101, 102, 103, 104 and a waveform generator which typically is a digital computer and which preferably is the same computer used for processing the ECL detected by the detection means.

In Fig. 14, each electrode/counterelectrode pair 101-104 is individually addressed by a pair of lines connected to the waveform generator. By way of example, lines 105, 106 access electrode/counterelectrode pair 101. An appropriate waveform may be applied by the waveform generator at any given time to any one or more of the pairs of lines connected to the various electrode/counterelectrode pairs.

To reduce the number of connections required to address the electrode pairs, alternatives to the direct connection scheme of Fig. 14 are provided. For example, a row-and-column accessing scheme is illustrated in Fig. 15 for electrically energizing some or all of the electrodes. In this scheme, one of the electrodes 201, 202 in each column of the plurality of electrode/counterelectrode pairs is connected to a common electrical conductor 205 on support 200, and each of the counterelectrodes in each row of the plurality of electrode/counterelectrode pairs is connected to conductor 207, 208 on the support 200. Conductors 205, 206 connect to connections C1, C2, respectively, at the edge of support 200 and

conductors 207, 208 connect to connections R1, R2, respectively. Each of these connections is then connected by a separate line to the waveform generator. As a result, in the configuration of Fig. 15, the number of required connections and signal lines from the waveform generator has been reduced from 8 to 4.

To enable rapid and sequential energizing of each electrode pair, a computer controlled switching device is beneficial. The configuration of Fig. 16 shows a plurality of electrodes connected to a first multiplexer 310. A plurality of counterelectrodes are connected to a second multiplexer 320. The first multiplexer is also connected to a first pole of a voltage source 330 that typically supplies the time varying electrical potential described *infra*. The second multiplexer is also connected to a second pole of the voltage source. Using addressing lines A0-A3 electrically connected to each of the multiplexers and connected to latch 340, a computer processor 350 can direct the multiplexers to selectively connect any or all of the first electrodes to the first pole of the voltage source, and any or all of the second electrodes to the second pole of the voltage source.

As shown in Fig. 17, a plurality of voltage sources are connected through separate sets of multiplexers to each of the electrodes. If a first electrical potential or range of electrical potentials is required at a particular electrode pair, the multiplexers 410, 420 associated with the voltage source 430 providing that potential are addressed by the computer processor 350, typically through a latch 340, thereby connecting that particular voltage source to the electrode pair in question. If a different electrical potential or range of electrical potentials is required for another electrode pair, the multiplexers 440, 450 associated with that different voltage source 460 are addressed by the computer processor, thereby connecting that voltage source through the associated multiplexers 440, 450 to the electrode pair.

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If the electrode array in this embodiment has at least a portion of the electrode pairs independently driveable, as shown in Fig. 14, or 15, for example, one electrode pair can be driven by one voltage source while another electrode pair is simultaneously driven with another voltage source. Alternatively, the two voltage sources of Fig. 17 can be replaced with a single voltage source connected to both sets of multiplexers in parallel, allowing two electrode pairs to be driven from the same voltage source.

Instead of a duplicate set of multiplexers for each voltage source as shown in Fig. 17, a plurality of voltage sources 520, 530 can be provided as shown in Fig. 18. These voltage sources can be connected through a computer controlled electrical switch 510 or switches to a single set of multiplexers 310, 320. As shown in Fig. 18, the computer would direct switch 510 to connect a particular voltage source to the multiplexers, and would also direct the multiplexers (by signalling their address lines A0-A3) to connect the selected voltage source to the particular electrode pair desired.

Alternatively, the electrical potential applied to each of the electrode pairs in any embodiment can be varied. This is of particular benefit when a cassette having a plurality of different binding domains is used. Such a cassette may require a different range of applied electrical potential at different binding domains. Several different embodiments capable of varying the electrical potential applied to each electrode are contemplated.

Advantageously, a computer controlled voltage source may be used. A computer controlled voltage source is one that can be addressed by a computer to select a particular electrical potential to be supplied. Alternatively it can be programmed to sequentially apply a particular range of electrical potentials over a predetermined time. In such a system, address lines electrically connected to the computer and the voltage source would allow the computer to program the voltage source to produce

the particular electrical potential to be applied to the electrode pair to be energized.

Additional methods for addressing the plurality of electrode pairs may also be used. For example, a plurality of reference electrodes may be placed in proximity to each of the plurality of electrode and counterelectrode pairs in order to sense the voltage applied thereto. In this way, additional control of the voltage waveform may be maintained.

Fig. 36 shows another embodiment of the invention; arrays of electrodes (3600, 3601) are supported on each of two surfaces (3602, 3603) separated by a pattern of gaps in an insulator 3604 (for example a plastic sheet with punched holes 3605. Each electrode may pass over a plurality of gaps. If a potential is applied between one electrode on each surface, current can only pass through a gap contacting both electrodes, thus limiting the location of any electrochemistry or ECL which may occur. In the preferred embodiment shown in the figure, the electrodes (3600, 3601) are arrays of lines on a support. The two sets of electrodes on the two surfaces are oriented perpendicular to each other. Gaps in the insulating sheet are located only at the intersection of the electrodes from each surface.

This embodiment has the advantage over individually addressed electrode pairs that less electrical leads are required.

In an alternate embodiment, the insulator 3604 is omitted and the surfaces are placed in close proximity so that only a narrow gap exists between the two surfaces. In this embodiment, a potential applied between are electrode on each surface will preferentially cause current to pass at the intersection of the electrode (i.e., where the distance between the electrodes is minimal) thus limiting the location of any electrochemistry or ECL which may occur.

5.5. LIGHT DETECTION

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The light generated by the triggered ECL emission is detected by an appropriate light detector or detectors positioned adjacent to the apparatus of the invention. The light detector may be, for example, film, a photomultiplier tube, photodiode, avalanche photo diode, charge coupled device ("CCD") or other light detector or camera. The light detector may be a single detector to detect sequential emissions or may be plural to detect and spatially resolve simultaneous emissions at single or multiple wavelengths of emitted light. The light emitted and detected may be visible light or may be emitted as non-visible radiation such as infrared or ultraviolet radiation. The detector or detectors may be stationary or movable. The emitted light or other radiation may be conducted to the detector or detectors by means of lenses, mirrors and fiberoptic light guides or light conduits (single, multiple, fixed, or moveable) positioned on or adjacent to the binding surface of the cassette or the detector may receive the light directly. In addition, the supports, PMAMS and electrode surfaces themselves can be utilized to guide or allow transmission of light.

The PMAMS may be formed on the surface of an array of light detectors so that each detector only receives light from one binding domain. The array of light detectors may be a CCD chip, and the binding domains may be attached (using standard coupling chemistries) to the surface of the semiconductor device.

Drops deposited on the binding domains, or on a near, second surface, can be used as microlenses to direct or control emitted light. Alternatively, a light detector can be oriented directly in front of the cassette; and various light focusing devices, such as parabolic reflectors or lenses may be employed instead of a light conduit to direct light from any of a plurality of binding domains to the detector. The light emitted from at least two discrete binding domains may be measured simultaneously or sequentially.

Error due to thermal drift, aging of the apparatus, or the electrical noise inherent in light detectors may be

controlled by a "chopper" means between the light measuring device and the binding domain being measured. The chopper can be any one of the common mechanical choppers well known to those of ordinary skill in the art, such as a spinning disk with slots or cutouts that allow light to pass. Alternatively, the light can be chopped by an LCD shutter, an array of LCD shutters, a solid state light valve or valves or the like. Alternatively, a planar array of LCD shutters or solid-state light valves such as those known in the art of optical computing may be used. These devices are preferably located between the plane of the cassette, and the light conduit (or conduits) or light focusing devices that direct light from the binding domains to the light detector. In an embodiment, a shutter is located above each of the binding domains. When using an LCD shutter or light valve, the shutters may be modulated at different frequencies to simultaneously provide different chopping rates for different light emitting binding domains. Using this technique, a plurality of different light signals may be superimposed and simultaneously measured by a single detector. An electronic band pass filter electrically connected to the light detector may then be used to separate the electrical single signal into several electrical components, each corresponding to one of the plurality of individual light components. By chopping the light, as above, or using other mechanism well known to the art, an AC light waveform is created that allows the DC noise component to be electronically filtered out.

Also, the ECL signal may be calibrated by comparison to results previously determined with standard reagents to correct for signal modulation due to reagent depletion.

5.6. ANALYSIS OF ECL SIGNALS

Signals arising from a given binding domain can have a range of values, and these values correlate with quantitative measurement to provide an 'analog' signal. In another technique a 'digital' signal is obtained from each domain to indicate that an analyte is either present or not present.

Statistical analysis is used for both techniques, and is particularly useful for translating a plurality of digital signals so as to provide a quantitative result. Some analytes, however, require a digital present/not present signal indicative of a threshold concentration. 'Analog' and/or 'digital' formats may be utilized separately or in combination. Other statistical methods can be utilized with PMAMS. For instance it is possible to create concentration gradients of PMAMS on a surface (Chaudhury et al., 1992, *Science* 256:1539-1541). This technique is used to determine concentrations through statistical analysis of binding over the concentration gradient. Multiple linear arrays of PMAMS with concentration gradients may be produced with a multiplicity of different specific binding reagents. The concentration gradients may consist of discrete binding domains presenting different concentrations of the binding reagents.

The presence of control assay systems on the binding surface of the cassette is also important to assure the uniformity of each analysis to control for signal variation (e.g., variations due to degradations, fluctuations, aging of the cassettes and other components, thermal shifts, noise in electronic circuitry and noise in the photodetection device, etc.). For example, multiple redundant binding domains (containing identical binding reagents or different binding reagents that are specific for the same analyte) for the same analyte may be utilized. In another example, analytes of known concentration are utilized or control domains of a PMAMS are covalently linked to a known quantity of an ECL label or a known quantity of ECL label in solution is used.

The assays conducted according to the invention will rapidly and efficiently collect large amounts of data that can be stored, e.g., in the form of a database consisting of a collection of clinical or research information. The data collected may also be used for rapid forensic or personal identification. For example, the use of a plurality of nucleic acid probes when exposed to a human DNA sample can be used for a

signature DNA fingerprint that can readily be used to identify clinical or research samples.

5.7. PREPARATION OF MULTI ELECTRODE ARRAYS

The electrodes may be broadly from 0.001 to 10 mm in width or diameter. In a preferred range the electrode pairs are from 0.01 to 1 mm in dimension (width or diameter or widest dimension depending upon the geometry of the electrode pairs).

Preferably, the electrodes are fabricated from suitable conductive materials, such as transparent metal films or semiconductors (e.g., gold or indium-tin oxide, respectively), as is well known to the art, for example, for the fabrication of liquid crystal displays and the like. In the assembled form of the cassette, sufficient space remains between the first and second supports to contain an analytic sample as, for example, a thin film or a wetted surface.

The electrodes may be fabricated from materials that contain carbon, carbon fibers, carbon nanotubes and/or aggregates of the above.

The electrodes may be fabricated from carbon fibrils. One or more individual fibrils and/or one or more aggregates of fibrils may be processed to form a larger aggregate (U.S. Patent No. 5,124,075).

This larger aggregate is a mat or mesh (hereinafter referred to as a "fibril mat") in which the fibrils may be entangled or interwoven. Fibril mats typically have a surface area between 50 and 400 M²/gram.

By way of example, a fibril mat may be used as a working electrode, a counter electrode or a reference electrode in analytical and/or preparative electrochemistry. In one example, the fibril mat is used as an electrode for electrochemiluminescence (ECL).

The binding domains of the PMAMS may be supported by a fibril mat. The PMAMS of the invention has a plurality of discrete binding domains, of which two or more may be identical

to each other or may differ. The fibril mat supports one or more binding domains.

One or more microfluidic guides may be used to prepare a plurality of binding domains on a fibril mat. Different or identical binding reagents may be present in a plurality of microfluidic guides and/or multiple distinct binding agents may be present in a microfluidic guide.

In Figs. 22A and 22B a plurality of microfluidic guides 2201, preferably in an array, are used to deliver, preferably concurrently, onto regions of the fibril mat 2200, drops containing the desired binding reagents, to form discrete binding domains 2202. The binding reagents form a bond with moieties present on the fibril mat. The binding reagents may adsorb non-specifically to the mat or dry on the surface.

The desired binding reagents are delivered to the fibril mat while suction filtration is applied to the mat. In this instance, the suction filtration draws none, some or all of the binding reagents into or through the mat, and in doing so, reduces the amount of lateral spreading of the binding reagents on the surface of the mat during the patterning process.

Fibril mats are prepared by compressing suspensions of carbon fibrils onto a substrate through which the liquid of the suspension may pass (e.g., a filter). Examples of filters that may be used to form fibril mats include filter paper, filters formed from polymeric (e.g., nylon) membranes, metal micromeshes, ceramic filters, glass filters, elastomeric filters, fiberglass filters and/or a combination of two or more of such filter materials. One of skill in the art of filtration would recognize that these materials are merely examples of the many possible materials suitable for filtration of suspensions of solids.

Fig. 23A and 23B illustrates an embodiment, in which fibril mats may be fabricated by suction filtration. A dispersion and/or suspension of carbon fibrils 2301 is filtered using a filter 2300 equipped optionally with a filter membrane 2303 and/or a filter support 2302. The suspension is filtered

using suction applied by a vacuum source 2305 to the filter by, for example, a filter flask 2306. A fibril mat 2304 collects on either or both the filter membrane 2303 and/or the filter support 2302. The fibril mat 2304, with or without the filter membrane 2303 may be removed from the filter.

In another embodiment, suspensions of fibrils are forced through a filter by use of pressure. In one example, pressure is exerted on a confined suspension of fibrils by compressing a confined layer of air and/or liquid above the suspension with a piston. In a specific example, the suspension of fibrils is confined in a syringe, the piston is a syringe plunger, and the filter is a disposable syringe filter (many such filters are well known to one of skill in the art).

Suspensions of fibrils are forced through a filter by capillary action or filtered by wicking of the suspension into or through a filter.

In another embodiment, individual fibrils or aggregates of fibrils are crosslinked covalently into mats. Fibrils derivatized with photosensitive moieties that polymerize when exposed to light are irradiated with light.

The filter may be used to trap the fibrils in its pores and so form a composite mat in which the filter acts as a support. In Fig. 24, a fibril mat 2400 may be prepared by passing a slurry of fibrils 2401, delivered by a source 2402, between two large rollers 2403. In this process, which may be analogous to processes found in the fabrication of paper or polymer sheets, the rollers force the liquid from the suspension and a large, continuous mat of fibrils is produced from which smaller mats may be cut.

Fibril mats may be freestanding (e.g., unsupported) or supported.

The rate of filtration can be varied to achieve desired properties in the mat. For example, properties that may be varied include uniformity or non-uniformity of structure, the extent of entanglement of the fibrils or aggregates of fibrils,

the thickness, the porosity of the mat, and/or combinations thereof.

Suspensions of carbon fibrils are confined and the liquid in which the fibrils are suspended is removed. In one example, the liquid in which the fibrils are suspended is allowed to evaporate. In another example, the liquid is removed by heating. In yet another example, the suspension is subjected to centrifugation, and the resulting liquid (e.g., the supernatant) is removed. In another example, the liquid is removed by evacuation.

The suspension may be placed on one or more of the filters described *supra*, and the suspension dried by evaporation. The suspension may be dried by heating or baking in an oven or the liquid may be removed by freezing and extracting the liquid. In yet another example, the liquid is removed by evacuation with a pump. Many other methods which are well known to one skilled in the art are available for removing liquids from a suspension.

Suspensions of fibrils suitable for forming fibril mats by filtration may be formed by dispersing one or more carbon fibrils in an appropriate liquid, quasi-solid or gel. Examples of appropriate liquids include but are not limited to water, ethanol, methanol, hexane, methylene chloride, buffered solutions, surfactants, organic solvents, solutions of containing biological media (e.g., as proteins, antibodies or fragments thereof, cells, subcellular particles, viruses, nucleic acids, antigens, lipoproteins, liposaccharides, lipids, glycoproteins, carbohydrates, peptides, hormones or pharmacological agents, solutions of small molecules, polymer precursors, solutions of acids or bases, oils and/or combinations thereof).

A suspension of fibrils may be prepared by dispersing carbon fibrils in an aqueous solution by means of sonication. In another embodiment, surfactant and or detergent may be added.

The fibril mat may have broadly a thickness between 0.01 μm and 10,000 μm .

In preferred embodiments, the fibril mat has a thickness between 1 μm and 100 μm . In particularly preferred embodiments, fibril mats range from 10 mm to 200 mm in width or diameter.

The fibril mat may be washed repeatedly and refiltered to remove residual materials remaining from the suspension.

Fibril mats prepared by filtration or evaporation are heated (e.g., in an oven) to remove residual liquid from the suspension not removed by filtration.

Successive filtration steps may be used to form mats of fibrils composed of one or more distinct layers that are either in contact with or in close proximity to one or more other layers. Layers may be distinguished by several properties, including but not limited to differences in the porosity, the density, the thickness, the distribution of sizes of individual fibrils and/or microscopic aggregates of fibrils, the type, number and/or size of fibril aggregates, the chemical derivatization of the fibrils (*vide infra*), and/or the presence of other matter attached to the fibrils.

Fig. 25, a multi-layered fibril mat 2500 is prepared by successive filtration steps. A 0.5 μm to 100 μm thick layer 2501 of plain fibrils forms the first layer; a 0.5 to 10 μm thick layer of fibrils 2502 that incorporate moieties such as poly-(ethyleneglycols) that resist adsorption of proteins and other molecules forms the second layer; a 0.5 to 5 μm thick layer 2503 that incorporates one or more binding domains (*vide supra*) forms the third layer. The binding domains contain one or more antibodies 2504, which may bind an analyte 2505. This antibody/analyte complex may bind a labeled antibody 2506. The label may be an ECL label. In other embodiments, the label may be one or more of a plurality of labels described elsewhere in this application. Such a multilayer mat may be freestanding or supported on one of a plurality of possible supports described above.

Multilayer mats may be formed in which there are combinations of layers, in which some or all of the layers may be different.

The filter used to form the fibril mat, the fibrils, and/or the fibril mat may be coated. In particular embodiments, the coatings are metallic. These coatings may be patterned such that certain portions are coated, and other portions are not. In one example, the coating is applied by electrodeposition. In another example, the coating is applied by electroless deposition.

The filter is coated with a metal, and the fibril is derivatized with a chemical functional group that forms a bond with said metal. The filter is a metal screen or metal sheet.

The fibril mat may be flat or deformed, regular or irregular, round, oval, rectangular, or one of many shapes, rigid or flexible, transparent, translucent, partially or fully opaque and may have composite properties or regions of different individual or composite properties.

The mat may be a disk or a piece taken from a sheet.

A plurality of fibril mats may be fabricated, preferably concurrently, and preferably in an array. In one example, an array of microfluidic guides forms a plurality of fibril mats on a support as described above. In another an array of filters, or a patterned filter (e.g., with regions of different porosity) is used to prepare an array of fibril mats.

A mask with an array of holes (e.g., a screen) is used to cover certain portions of a filter or support, and a plurality of discrete fibril mats are made concurrently by either filtration and/or evaporation.

Fibril mats may have a density from 0.1 to 3.0 grams/cm². The mat may have variable density. For example, mechanical force or pressure may be applied to the mat at different times to increase or decrease the density.

Fibril mats may have pores. These pores may extend partially and/or fully through the mat or may be part of a

network or pores. These pores may have dimensions ranging broadly from 50 Å to 1000 μm. In a preferred embodiment, the fibril mat has pores with dimensions ranging from 200 Å to 500 Å. The porosity of the mat may depend on the density of the mat, among other factors.

The porosity of the mat may be constant throughout the mat or may increase or decrease as a function of the position in the mat. The fibril mat may have a wide variety of pores of different size distributed in a disorganized and/or random manner.

The fibril mat may contain distinct regions of different porosity. For example, the fibril mat may have one or more layers, each having a different porosity. The fibril mats may have columns of different porosity that run through the mat.

The porosity of the mat may be varied by including different amounts of aggregates of carbon fibrils, where aggregates have different size, shape, composition, or combinations. In a particular example, a mat is prepared from individual fibrils, CC fibrils (described *supra*) and BN fibrils (described *supra*), or different combinations. For example, the fibril mat may have some pores that are large enough to pass objects as large as biological cells, some pores that can pass biological media as large as proteins or antibodies, some pores that can pass only small (< 1000 molecular weight) organic molecules, and/or combinations thereof.

The porosity of the mat may be such that one or more molecules, liquids, solids, emulsions, suspensions, gases, gels and/or dispersions can diffuse into, within and/or through the mat. The porosity of the fibril mat is such that biological media can diffuse (actively or passively) or be forced by some means into, within and/or through the mat. Examples of biological media include but are not limited to whole blood, fractionated blood, plasma, serum, urine, solutions of proteins, antibodies or fragments thereof, cells, subcellular particles, viruses, nucleic acids, antigens, lipoproteins, liposaccharides,

lipids, glycoproteins, carbohydrates, peptides, hormones or pharmacological agents. The fibril mat may have one or more layers of different porosity such that material may pass through one or more layers, but not through other layers.

The fibril mat is supported by or on another material. By way of example, the supporting material may be a metal, plastic, polymer, elastomer, gel, paper, ceramic, glass, liquid, wax, oil, paraffin, organic solid, carbon or a mixture of two or more of each. The material may be solid or liquid. If it is solid, it may contain one or a plurality of holes or pores. In specific examples, the support may be a metal mesh, a nylon filter membrane or a filter paper. The support may be a conductor, a semiconductor and/or an insulator.

In an embodiment disclosed in U.S. Patent Nos. 5,304,326 and 5,098,771, fibrils may be dispersed in another material. For example, fibrils may be dispersed in oils, waxes, paraffin, plastics (e.g., ABS, polystyrene, polyethylene, acrylonitrile, etc.), ceramics, teflon, polymers, elastomers, gel, and/or combinations thereof. Dispersions of fibrils in other materials are conducting. Dispersions of fibrils in other materials may be molded, pressed, formed, cast, spun, weaved, and/or thrown so as to form objects of a desired shape and/or form. The fibril mat may incorporate another material, for example thin fibers, shards, or balls of metal to increase the conductivity of the mat. In another example, the fibril mat may incorporate other carbon, glass and/or metal fibers of varying size, shape and density to create a different porosity than can be achieved with fibrils alone. In another aspect, the mat may incorporate magnetic beads (for example, DYNAL beads). In the latter example, the beads may either serve to change a property of the mat, or may themselves be used as supports to immobilize binding domains.

Other carbon fibers (e.g., carbon nanostructures, carbon nanotubes, buckminsterfullerenes, buckytubes, fullerenes, or combinations thereof) may be used in place of carbon fibrils.

Carbon fibrils may be prepared with chemical functional groups attached covalently to their surface. As described in International Publication No. WO 90/14221, these chemical functional groups include but are not limited to COOH, OH, NH₂, N-hydroxy succinimide (NHS)-esters, poly-(ethylene glycols), thiols, alkyl ((CH₂)_n) groups, and/or combinations thereof. These and other chemical functional groups can be used to attach other materials to the surface of fibrils.

Certain chemical functional groups (e.g., COOH, NH₂, SH, NHS-esters) may be used to couple other small molecules to the fibrils. There are a plurality of possible combinations of such chemical functional groups and small molecules.

In many embodiments, NHS-ester groups are used to attach other molecules or materials bearing a nucleophilic chemical functional group (e.g., an amine). In a preferred embodiment, the nucleophilic chemical functional group is present on and/or in a biomolecule, either naturally and/or by chemical derivatization. Examples of suitable biomolecules include but are not limited to amino acids, proteins and functional fragments thereof, antibodies, binding fragments of antibodies, enzymes, nucleic acids, and combinations thereof. This is one of many such possible techniques and is generally applicable to the examples given here and many other analogous materials and/or biomolecules. In a preferred embodiment, reagents that may be used for ECL may be attached to the fibril via NHS-ester groups.

An antibody that can be used in an ECL assay can be attached to one or more fibrils or a fibril mat by covalent bonds (e.g., reaction with an NHS-ester), by reaction with an appropriate linker (vide supra), by non-specific binding, and/or by a combination thereof. Nucleic acids and/or cells can be attached to fibrils or fibril mats by covalent links to NHS-esters attached to the fibrils.

It may be desirable to control the extent of non-specific binding of materials to fibrils and/or fibril mats. Simply by way of non-limiting examples, it may be desirable to

reduce or prevent the non-specific adsorption of proteins, antibodies, fragments of antibodies, cells, subcellular particles, viruses, serum and/or one or more of its components, ECL tags (e.g., $\text{Ru}^{\text{II}}(\text{bpy})_3$ and $\text{Ru}^{\text{III}}(\text{bpy})_3$ derivatives), oxalates, trialkylamines, antigens, analytes, and/or combinations thereof. In another example, it may be desirable to enhance the binding of biomolecules.

One or more chemical moieties that reduce or prevent non-specific binding may be present in, on, or in proximity to one or more fibrils, one or more fibril aggregates and/or a fibril mat. Non-specific binding is controlled by covalently attaching PEG moieties to one or more fibrils and/or a fibril mat. Charged residues (e.g., phosphates, ammonium ions) may be attached covalently to one or more fibrils or a fibril mat.

Materials used in the support, electrode and/or binding domain may be treated with surfactants to reduce non-specific binding. For example, fibrils or fibril mats may be treated with surfactants and/or detergents that are well known to one of ordinary skill in the art (for example, the Tween series, Triton, Span, Brij). The fibrils or fibril mats are washed, soaked, incubated with, sonicated in, and/or a combination thereof with solutions of surfactants and/or detergents. Solutions of PEGs and/or molecules which behave in similar fashion to PEG (e.g., oligo- or polysaccharides, other hydrophilic oligomers or polymers) ("Polyethylene glycol chemistry: Biotechnical and biomedical applications, Harris, J.M. Editor, 1992, Plenum Press) may be used instead of and/or in conjunction with surfactants and/or detergents.

Undesirable non-specific adsorption of certain entities such as those listed above may be blocked by competitive non-specific adsorption. This competitive binding species might be bovine serum albumin (BSA) immunoglobulin G (IgG).

Non-specific binding of the ECL-TAG may be reduced by chemical modification of the TAG. For example, the TAG may be modified so as to increase its hydrophilicity (e.g. by adding

hydrophilic, polar, hydrogen bonding, and/or charged functional groups to the bipyridyl ligands in Ru(bpy₃)) and thus reduce non-specific binding of the TAG to other surfaces.

It may be desirable to immobilize biomolecules or other media to fibrils or fibril mats. One may attach antibodies, fragments of antibodies, proteins, enzymes, enzyme substrates, inhibitors, cofactors, antigens, haptens, lipoproteins, liposaccharides, cells, sub-cellular components, cell receptors, viruses, nucleic acids, antigens, lipids, glycoproteins, carbohydrates, peptides, amino acids, hormones, protein-binding ligands, pharmacological agents, and/or combinations thereof.

It may also be desirable to attach non-biological entities such as, but not limited to polymers, elastomers, gels, coatings, ECL tags, redox active species (e.g., tripropylamine, oxalates), inorganic materials, chelating agents, linkers etc. to fibrils.

One or more or a plurality of species may become bound non-specifically (e.g., adsorb) to the surface of the fibrils or fibril mat.

Biological molecules or other media can be attached to fibrils or fibril mats by non-specific adsorption. The extent of non-specific adsorption for any given fibril, fibril mat and/or biomolecule will be determined by certain properties of each. Certain chemical functional groups or biological moieties present on fibrils may reduce or enhance non-specific binding. The presence of hydrophobic and/or hydrophilic patches on the surface of a protein may enhance or reduce non-specific binding of the protein to fibrils or fibril mats. Hydrophilic and/or hydrophobic patches are utilized to control non-specific binding in controlled areas.

Fibrils can be derivatized with alkyl (CH₂) chains and/or carboxylic acid groups to enhance non-specific binding of biological molecules or media or other materials.

Fig. 26 illustrates the above embodiment schematically in the case of a single fibril. A fibril 2600 is derivatized

with alkyl chains 2601. Biomolecules 2602, 2603, and 2604 bind non-specifically to the alkyl chains. Polymer/elastomer 2605 is also bound.

Underivatized fibrils, fibril aggregates and/or fibril mats are used for immobilization of biomolecules, biological media, and other materials by non-specific binding.

The ECL TAG contains charged residues. The ECL TAG is made to be selectively attracted to a support and/or electrode. For example, a derivatized ECL TAG which has a net negative charge may have relatively low affinity for an electrode at more reducing potential and then has higher affinity for the electrode as the electrode potential becomes more oxidizing. The affinity of the ECL tag and/or binding reagents to the electrode is made to modulate (e.g., to decrease affinity during binding and/or washing steps and to increase the affinity of the ECL TAG and/or binding reagents so as to increase the effective potential felt by the ECL TAG during an ECL reading).

In Fig. 28 molecules (both biological and non-biological) may be attached to fibrils by means of a covalent bond. Fibrils 2800 bearing an NHS-ester chemical functional groups may form covalent bonds 2801 to biomolecules or biological media 2802, 2803. These biological media may use an amino group to form a covalent bond by reaction with the NHS-ester group. Polymer 2808 is immobilized. One of ordinary skill in the art would recognize the generality of NHS-ester groups as coupling agents for molecules and would be able to select both the appropriate biomolecules and the appropriate reaction conditions to achieve immobilization.

A pair of moieties and/or molecules "M1 " and "S1", of which one or more is attached to a fibril, exhibit a mutual affinity or binding capacity. M1/S1 may be antibody/antigen, antibody/hapten, enzyme/substrate, enzyme/cofactor, enzyme/inhibitor, lectin/carbohydrate, receptor/hormone, receptor/effector, nucleic acid/nucleic acid, protein/nucleic acid, virus/ligand, cell/cellular receptor, etc. Many

combinations of "binding pairs" M1/S1 and would be able to select combinations appropriate to achieve the desired binding. Either or both M1 and S1 may be attached to one or more fibrils.

Figs. 27 and 28 illustrate some of the many possible configurations that are possible with this embodiment. In Fig. 27, a fibril 2700 derivatized with alkyl chains 2701 non-specifically binds a molecule 2702 that has a mutual affinity or binding capacity for another molecule 2703. Molecule 2703 is also attached to another molecule 2704. A blocking molecule 2705 may be non-specifically adsorbed to the fibril. A blocking polymer 2706 and/or a polymer 2707 which has a ligand (2708) that has an affinity for a molecule 2709 are non-specifically adsorbed.

In Fig. 28, a fibril 2800 is covalently linked via 2801 to biomolecules 2802 and 2803, and a linker molecule 2804. The linker molecule 2804 has a mutual affinity or binding capacity for another biomolecule 2805. Biomolecule 2803 has a mutual affinity or binding capacity for another linker molecule 2806, which is covalently linked to 2807. Polymer 2808 with a ligand 2812 that is specific for a binding partner 2809 is covalently linked to a fibril. Blocking molecules (e.g. BSA) 2811 and blocking polymers 2810 are covalently attached.

A fibril may be derivatized with biotin and/or a biotinylated linker and avidin and/or streptavidin may bind to this linker. Avidin and/or streptavidin may be bound to the fibril, and a biotinylated antibody and/or protein may bind. Avidin and/or streptavidin may be immobilized on the fibrils by either non-specific binding, covalent bond, another or the same coupling pair, or a combination thereof. The use of (strept)avidin and biotin as "binding pairs" is a widely applied method of attaching biomolecules or biological media to other materials and is well known to those skilled in the art (Spinke et al., 1993, Langmuir 9:1821).

A binding pair may be a monoclonal antibody and an antigen that binds to this antibody.

Multiple binding pairs (e.g., M1/S1/M2) may form. M1 is a monoclonal antibody, S1 is an antigen to M1, and M2 is an antibody that binds to S1. This complex may constitute an antibody/antigen/antibody "sandwich" complex (such antibodies may or may not be monoclonal). M2 may be an antibody tagged with an ECL-active tag (vide supra), a fluorescent label, a radioactive label, an enzymic tag, and/or combinations thereof.

M1 may be a moiety that can complex with a metal, metal ion, or organometallic compound (a "chelating agent") and S1 is a metal, metal ion, or organometallic compound (a "chelate") that forms a complex with M1, and M2 is a moiety on a biological molecule that binds to the M1/S1 complex (Gershon and Khilko, 1995, Journal of Immunological Methods, 7371).

The fabrication of metallic electrode patterns and conductive elements to distribute electrical current to such electrodes on a surface is carried out by methods well known to the art (see, e.g., Leventis et al., U.S. Patent No. 5,189,549). The preparation of metal films on transparent surfaces is used to produce liquid crystal displays and is readily adapted to the preparation of electrodes according to the invention. Haneko, 1987, Liquid Crystal TV Displays, Principles and Applications of Liquid Crystal Displays, KTK Scientific Publishers, Tokyo, D. Reidel Publishing. Transparent electrode surfaces may also be prepared, for example, according to the method of DiMilla et al., 1994, J. Am. Chem. Soc. 116(5):2225-2226. 0.5 nm of titanium and 5 nm of gold are deposited on transparent substrates (glass or plastic). A thin gold layer as prepared by the method of DiMilla, *supra* may be used to prepare a transparent electrical structure by the method of Kumar *supra*. Modifications of this procedure to increase the thickness of the conductor layers for improved current carrying capacity while preferably maintaining transparency are desirable and readily apparent to the ordinary artisan. Such techniques may be used to prepare electrode surfaces that are aligned with or in proximity with discrete binding domains of a PMAMS.

In addition, the films and/or monolayers may be composed of moieties which facilitate the transfer of electrical potential from the electrode surface to the ECL label, rather than using insulating moieties (e.g., alkyl chains) as taught by Zhang and Bard. For example, pi orbital overlap in extensively conjugated systems can be used for electron transfer. Such pi orbital electron transfer is provided by poly-pyrrole or other conjugated rings or double bonded structures.

Oligonucleotides may be utilized to modulate electron transfer. For example, overlapping pi bonds in double stranded DNA may be utilized to increase electron transfer rates. Oligonucleotides bound to an electrode surface can be utilized as a binding agent in a binding domain. Upon binding a complementary oligonucleotide sequence a double strand with organized overlapping pi bonds is formed. In a particular embodiment, a first or primary immobilized (e.g., covalently linked to a support) oligonucleotide is ECL labeled. In another embodiment a secondary complementary oligonucleotide or oligonucleotide of partially complementary sequence to the primary oligonucleotide is ECL labeled. A tertiary oligonucleotide complementary to or partially complementary to the secondary oligonucleotide is labeled (e.g., a sandwich assay). Branched oligonucleotide chains may also be utilized. A variety of oligonucleotides and/or oligonucleotide mimics can be utilized (e.g., oligonucleotides with modified bases and/or modified backbones containing for example nitrogen and/or sulfur). Differential studies may be performed. Variable stability of pi overlap in oligonucleotides and/or oligonucleotide complexes may be monitored through modulation of electron transfer. The signal (e.g., ECL light generated and/or impedance measurements) generated from a pi bond stabilized ECL labeled double helical oligonucleotide pair may be correlated against the signal and/or expected signal from a more disordered single stranded oligonucleotide. The variation in ECL signal between a fully complementary ECL labeled double stranded

oligonucleotide and a partially complementary ECL labeled double stranded oligonucleotide may be correlated. Additionally, oligonucleotide complexes of multiple oligonucleotides may be utilized. For example, triple helices may be employed.

Modulation of electron transfer rates may be measured using ECL detection as well as electronic means. ECL labels may be covalently linked to oligonucleotide strands and/or non-covalently associated (e.g., intercalated). DNA may be coupled to the electrode without the use of a linker (e.g., adsorption of 5' thiolated DNA on gold) or with a short (< 10 atom) linker to ensure low resistance to electron transfer from the DNA to the electrode. A linking chain may be used that can efficiently transport electrons from the electrode to the DNA strand (e.g., a polyacetylene chain).

A mixed monolayer and/or film may be used in which at least one constituent of the monolayer or film, as the case may be, facilitates the transfer of electrical potential. Alternatively, a molecule or particle that facilitates the transfer of electrical potential is adsorbed to the monolayer or film. As examples of the foregoing, the pi conjugated monolayers and/or conducting micro-particles which adsorb to and/or are approximate to the electrode surface, may be used. Patterned regular gaps are created in the monolayer and or film. By utilizing controlled patterns of gaps in an ordered substantially perpendicular SAM composed of long chain alkane thiols (i.e., insulating) to which ECL labels have subsequently been attached, the effective potential imposed at the ECL labels can be controlled. For example, Fig. 11 shows a cassette 1200 formed of a single support 1202 with a metallic layer 1204, a SAM pattern 1206 and gaps 1208 between the SAM patterns.

ECL labeled proteins may be non-covalently linked to a monolayer surface. An ECL labeled protein may adsorb to the surface of a methyl terminated alkane thiol derivatized gold surface. The gold surface may act as the working electrode or the counter electrode. A plurality of binding domains may be

incorporated on a single support as is illustrated in Figs. 11-13. In preferred embodiments the binding domains contain labelled and/or unlabelled proteins and/or nucleic acids and/or cells and/or chemical species.

Alternatively, the length of the components of the monolayer (e.g., the length of the alkane chain in alkane thiol monolayers) may be varied to control the effective potential at the exposed surface of the monolayer.

Broadly, alkane thiols may have carbon chains of length between 1 carbon and 100 carbons. In a preferred embodiment the carbon length of the alkane thiol contains between 2 and 24 carbons. The carbon chain length of the alkane thiol is between 2 and 18 carbons. The carbon chain length is between 7 and 11 carbons. Such alkane thiols may have a variety of head groups exposed to the assay media including methyl groups, hydroxy groups, amines, carboxylic acids, oligo (ethylene glycols), phosphate groups, phosphoryl groups, biotin, nitrilotriacetic acid, glutathione, epoxides, dinitrophenyl, and/or NHS esters. Other head groups include ligands commonly used for the purification and immobilization of recombinant fusion proteins (e.g., Sassenfeld, 1990, TIBTECH 8:88-93). The binding domains may be derivatized to varying degrees to achieve varying densities of binding reagents. For example, different densities of activatable chemistries may be used and/or derivatization may be carried out to varying extents. Mixed chemistries may be utilized to create desired binding densities. Mixed monolayers may be utilized to control the density of activatable groups and/or binding reagents. The density of binding groups is controlled to optimize the ECL signal to noise ratio. The total number of binding sites within a binding domain(s) is controlled to optimize the intensity of the ECL light signal with respect to other ECL light signals from other binding domain(s) whether such ECL light signals are detected sequentially or simultaneously and/or with respect to the light detection means.

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said multiplicity of ECL signals may be utilized to increase signal to noise by, for example, filtering certain ECL signals of a series. Further, multiple electronic potential waveform pulses may be utilized to reduce undesirable modulation of signal due to non-specific binding. Electronic potential may be applied to prevent non-specific binding of certain charged species. Additionally, electronic potential may be applied so as to promote the localization near a binding domain(s) of certain analytes or chemical species of interest. The voltage waveform applied supplies large over-potential (e.g., higher potential than is required to generate ECL). Over-potentials may be utilized to modulate ECL signals in a voltage wave series or in a single voltage wave pulse. Additionally, over-potentials may be utilized to modulate the ECL reaction kinetics and/or modulate the binding potential chemically and/or physically. Further, one or more voltage waveforms and/or other electronic probes known to those skilled in the art may be utilized to assess and/or correlate and/or extrapolate information on the quality and/or electronic properties of an electrode(s).

Preferably, the efficiency of the ECL reaction may be enhanced by extending the working electrode surface area by providing additional conducting means in contact with the electrodes. Projections or extensions from the electrode (e.g., wires or whiskers) of conducting materials or conducting particles may be used to increase the electrode surface area, such that the electrical field and more closely approaches the ECL label. Alternatively, indentations or wells in the electrode structures may serve the same purpose.

In particular, conductive particles may fill the gaps on the electrode surface and/or cover the support or monolayer so that the electrical field around the ECL label is increased in absolute magnitude, as shown by Fig. 12. These conductive particles extend the electrode surface area and thereby increase the efficiency of the ECL reaction. Fig. 12 shows a cassette 1300 having a support 1302 bearing a patterned SAM 1306 on a

metallic layer 1304 and indicates conducting micro-particles filling in the gaps (e.g., 1208 in Fig. 11) and extending above the metallic surface between the SAM patterns. For magnetic conducting particles, a magnet or magnetic field may be used to draw the particles to the surface. The conductive particles may also be used as described to extend the electrical potential between the electrode surfaces and the binding domain of a PMAMS with two approximated supports. In Fig. 8, the cassette 900 consists of a first support 902 that has a multi array of electrodes, and a second support 904 that has a PMAMS. Conducting micro-particles 906 are positioned between the opposing surfaces in order to extend the electrical potential toward the ECL label on the binding domains (not shown).

Alternatively, conductive polymers are grown from the exposed gaps on the electrode surface to facilitate extending the electrical potential around the ECL label of the sample as shown by Fig. 13. Fig. 13 shows a cassette 1400 having a support 1402 bearing a metallic layer 1404 on a patterned SAM surface 1406. Conductive polymers 1408 are grown over the SAM surface to extend the electrical field provided by a multi array of electrodes (not shown) to binding domains (not shown) on the SAM surface. The conductive polymers may also be used as described to extend the electrical potential between the electrode surfaces and the binding domains of a PMAMS of two approximated supports as illustrated by Fig. 7. In Fig. 7, the cassette 800 consists of approximated supports 802 and 804. Conductive polymers 806 are grown between the opposed surfaces so as to extend the electrical potential toward the ECL label on the binding domain (not shown).

Fig. 9 illustrates a cassette 1000 formed with a first support 1002 having a multielectrode array, a second support 1004 having a PMAMS binding surface, wherein conductive projections (1006) (e.g., fine wire or other protrusions) of the working electrode extend the electrical field around the ECL label in the PMAMS binding domains.

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The electrode pairs may be created in a variety of configurations. The simplest configurations, depicted in the figures accompanying this disclosure, are made of metal and/or metal oxide films and/or semiconductor films applied on a non-conducting planar surface. The electrodes of these electrode pairs preferably define between them a region of relatively constant width thereby providing a relatively constant electrical field.

Other configurations of the electrodes are provided. Several of these configurations are shown in plane views in Figs. 19(a)-(e). Fig. 19(a) shows an inter-digitated comb-like electrode pair. In this structure, each electrode has a plurality of digits extending from the conductor making a comb-like shape. The electrode and counterelectrode pair may be positioned adjacent to a binding domain, or a binding domain may be positioned between an electrode and counterelectrode. Fig. 19(b) shows a pair of concentric electrodes, one circular and one semicircular. Fig. 19(c) shows two semicircular electrodes with their straight edges facing each other. Fig. 19(d) shows a pair of rectangular electrodes. Fig. 19(e) shows a pair of interdigitated electrodes having complementary opposing curved surfaces to form a sinuous gap therebetween.

The electrode/counterelectrode pairs may also be formed into specific shapes complementary to shapes on the PMAMS binding surface for alignment purposes. Exemplary shapes are shown in Fig. 6B. A support 712 bearing electrode pairs 714-720 is shown. The electrode pairs may be, e.g., circular 714, interdigitated 716 triangular interdigitating 718 or multi electrode interdigitating 720.

In the embodiments shown in Figs. 14-19 discussed *supra*, the electrode pairs are located on a single support. Alternatively, the electrode pairs are located on first and second opposing supports as shown by Fig. 2.

5.8. CASSETTES

Cassettes contain one or more supports of the invention. Cassettes may include a plurality of binding domains and one or more working electrodes.

Fig. 2 depicts a cassette where each of plural binding domains 30 on support 26 are adjacent to a different one of plural electrodes 32. Counterelectrodes 38 are formed on a second support 28. An ECL assay is conducted as previously described by placing a sample on binding domains 30 and then moving together supports 26 and 28 so that counterelectrodes 38 are each adjacent to each of binding domains 30 and an ECL reaction may be triggered as described above by waveform generator means 39, via a lead 34, and an ECL signal detected and recorded by light detector means 40, wire 41, and digital computer means 42.

Fig. 3 illustrates a cassette where each of plural binding domains 48 has a different one of plural electrode/counterelectrode pairs 50 adjacent thereto on support 44. Support 46 may optionally be placed adjacent to support 44 so that support 46 forms a sample containing means adjacent to plural binding domains 48 and plural electrodes 50. Thus, an ECL reaction may be triggered via electrical connection 52 by waveform generator means 54, and an ECL signal detected by light detector means 56 and recorded and analyzed by digital computer means 58.

A cassette is provided that contains one or more pairs of supports as shown in Fig. 21, each pair of supports being situated so that the surface of a first support 1501 that contains binding domains faces the surface containing binding domains on the second support 1502, in which each surface contains electrodes 1504 and binding domains 1506; such that each binding domain on the first support faces and is aligned with an electrode on the second support, and each binding domain on the second support faces and is aligned with an electrode on the first support.

Fig. 4 illustrates a cassette wherein ECL electrodes are optional. Binding domains 64 on support 60 are contacted with a sample suspected of containing an analyte. Regions 66 on support 62 contain reaction medium for detecting or measuring an analyte of interest or for carrying out a desired reaction. Support 60 and Support 62 are brought together so that binding domains 64 and regions 66 are contacted and the presence of an analyte or reaction product is determined by a reporter system, e.g. a colorimetric chemiluminescent or fluorescent signal that may be detected by photodetector means 68 and recorded and analyzed by digital computer means 70.

In a preferred embodiment, a cassette or apparatus of the invention comprises a means for sample delivery onto the plurality of discrete binding domains (see, e.g., element 1 on Fig. 1 of U.S. Patent 5,147,806; element 1 on Fig. 1 of U.S. Patent 5,068,088; each of which is incorporated by reference in its entirety). The means for sample delivery can be stationary or movable and can be any known in the art, including but not limited to one or more inlets, holes, pores, channels, pipes, microfluidic guides (e.g., capillaries), tubes, spigots, etc. Fluids can be moved through the system by a variety of well known methods, for example: pumps, pipettes, syringes, gravity flow, capillary action, wicking, electrophoresis, pressure, vacuum, etc. The means for fluid movement may be located on the cassette or on a separate unit. The sample can be placed on all of the binding domains together. Alternatively, a sample may be placed on particular binding domains by a capillary fluid transport means. Alternatively, samples may be placed on the support by an automatic pipetter for delivery of fluid samples directly to the PMAMS on a support, or into a reservoir in a cassette or cassette holder for later delivery directly to the binding surface.

Supports may be prepared from materials including but not limited to, glass, plastic, ceramic, polymeric materials, elastomeric materials, metals, carbon or carbon containing materials, alloys, composite foils, silicon and/or layered

materials. Supports may have a wide variety of structural, chemical and/or optical properties. They may be rigid or flexible, flat or deformed, transparent, translucent, partially or fully reflective or opaque and may have composite properties, regions with different properties, and may be a composite of more than one material.

Reagents for conducting assays may be stored on the cassette and/or in a separate container. Reagents may be stored in a dry and/or wet state. In one embodiment, dry reagents in the cassette are rehydrated by the addition of a test sample. In a different embodiment, the reagents are stored in solution in "blister packs" which are burst open due to pressure from a movable roller or piston. The cassettes may contain a waste compartment or sponge for the storage of liquid waste after completion of the assay. In one embodiment, the cassette includes a device for preparation of the biological sample to be tested. A filter may be included for removing cells from blood. In another example, the cassette may include a device such as a precision capillary for the metering of sample.

The plurality of binding domains and the plurality of electrodes/counterelectrodes on the supports are typically placed in registered proximity to one another by mechanical means, e.g., by using guide posts, alignment pins, hinges (between each support) or guide edges. Optical guide means may be used to position both supports and electronic means utilizing optical guide marks defined on the supports. Other systems using electrical or magnetic registration means are also available.

The supports of the cassette may be configured so as to protect the electrode pairs from contact with the sample until required to trigger an ECL reaction. For example, the electrodes may be kept separate from a binding domain surface until electrode contact with the sample is required by using various mechanical means such as a removable electrode protective means.

A cassette or apparatus of the invention comprises reference electrodes, e.g., Ag/AgCl or a saturated calomel electrode (SCE).

The supports may be held together by clips, adhesive, rivets, pins or any other suitable mechanical attachment. They may also be held together by the surface tension of a liquid sample or by a compression means removably placed on opposite sides of the two supports.

The cassette may also comprise more than two supports, with, for example, alternating layers of binding domains and electrodes or multiple supports comprising both a binding surface and an electrode surface on a single support. This will form a three dimensional array of ECL analysis cells. All of the foregoing components of the cassette are transparent, except, optionally, some areas between the binding domains. For example, multiple transparent binding surfaces, electrode surfaces, and supports may be stacked.

The first and second supports may be flat and opposed to define a sample-holding volume therebetween. Alternatively, the first and second support layers may be configured in other suitable shapes including spheroidal, cuboidal, cylindrical, provided that the two supports, and any other components thereof, conform in shape. For example, Fig. 10 shows a cassette 1100 formed from two adjacent non-planar supports 1102 and 1104. Each support has a surface complementary to the other in conformation. Either support may have a PMAMS surface or a multi electrode array or both. One or both of the supports may be elastomeric so as to conform to the shape of the other support. The supports or the cassettes may also be prepared in a precut format, or dispensed in a suitable length from a roll dispenser. The cassette may further include sample receiving means such as a sample-holding volume and sample distribution grooves, channels, indentations and the like.

Fig. 37 shows a cassette where binding domains (3702) in and/or on a matrix (3703) are presented on a surface (3701).

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A second surface (3700) supporting a working electrode (3704) and a counter electrode (3705) is placed so that the binding domains are in close proximity to the working electrode. Under conditions which lead to light generation from ECL label bound to the binding domains, light may be detected through either or both surfaces. An array of light detectors (3706, e.g., a CCD array, an intensified CCD array, or an avalanche photodiode array) is used to simultaneously measure the plurality of light signals from each of the binding domains. The light detector array images the light generated from binding domains. Lenses, reflectors and/or optical guides may be utilized to enhance imaging. In other examples, light detected from zones or regions of light detectors (e.g., a light detecting pixel(s)) is correlated to a binding domain(s). Image analysis may be used to aid in the correlation of detected light with binding domains. In one favored embodiment, the surface is elastomeric or compliant and therefore capable of making intimate contact with the electrode surfaces. The binding domains are linked to polymers capable of carrying ionic currents from the counter electrode to the working electrode. In a more favored embodiment, the objects are water-swollen polymers capable of carrying an ionic current from the counter electrode to the working electrode.

Fig. 38 shows a cassette where binding domains (3805, 3806, 3807) are presented on the surfaces of distinct objects (3808, 3809, 3810) supported on the counter electrode (3800). A working electrode (3801) is placed in proximity to the surface of the objects. Under conditions which lead to ECL from TAGged groups bound to the binding domains, light may be detected through either or both of the electrodes (if one or both of the electrodes is transparent or semi-transparent) and/or from the side. An array of light detectors (3802) is used to simultaneously measure the plurality of light signals from each of the binding domains. The objects may be elastomeric and/or compliant and are therefore capable of forming intimate contact

with the working electrode. The objects may be polymers capable of carrying ionic currents from the counter electrode to the working electrode. The objects may be water-swollen polymers capable of carrying an ionic current from the counter electrode to the working electrode.

A transparent support containing one or more binding domains is brought into contact with a fibril mat electrode. Reagents may be flowed either between the support/binding domains and the fibril mat, or through the mat to the binding domains. Light may pass from the binding domains, through the transparent support to a detector.

In another preferred embodiment, an electrode is coated with an optically translucent or transparent layer of carbon fibrils, so as to increase the effective surface area of the electrode.

Advantageously, the PMAMS supports and/or cassettes of the invention may be packaged as kits. The kit comprises one or more PMAMS supports prepared according to the invention for conducting ECL reactions including assays, controls and the like. Reagents may be optionally included in the kit, including control reagents, ECL assay and calibration reagents and the like. A reagent mixture may be included which contains a plurality of binding reagents specific for a plurality of different analytes.

5.9. APPARATUS FOR CONDUCTING ECL REACTIONS

In one embodiment, the PMAMS on supports, and cassettes containing the same, are designed to be inserted into an apparatus, that contains means for applying one or more test samples onto the PMAMS binding domains and initiating a plurality of ECL reactions. Such apparatus may be derived from conventional apparatus suitably modified according to the invention to conduct a plurality of ECL assays based on a support or cassette. The invention provides various apparatus adapted to carry out ECL assays using each of the specific embodiments of PMAMS described in the Sections hereinabove. An apparatus for conducting ECL reactions is disclosed by Zoski et al. (U.S.

Patent No. 5,061,445). Modifications required include the provision for support and/or cassette handling, multiple sample delivery, multiple electrode addressing by a source for a voltage waveform and multiple ECL signal acquisition and processing.

Elements of illustrative apparatus in accordance with the invention are shown in Fig. 6A. Such apparatus 700 comprises upper and lower supports 702, 704 and an electrode guard 710. The upper support bears a plurality of electrode/counterelectrode pairs (not illustrated). The lower support bears the binding domains 706. The apparatus is capable of removing the electrode guard from the cassette and positioning the electrode/counterelectrodes to contact the analyte bound to the binding domains. A reagent or fluid flow space 708 is adjacent to the support bearing the binding domains. The apparatus is also capable of simultaneously or sequentially sending an identical or individually determined voltage wave to each of the plurality of electrode/counterelectrode pairs to trigger ECL reactions in the cassette and then measuring the emitted ECL radiation, by a photon detector, e.g., light detector means. The apparatus can further comprise temperature control means for maintaining the temperature of the support and/or cassette, or the environment thereon and adjusting the temperature as needed to optimize ECL reaction conditions. Temperature control means are preferably heating and cooling means, e.g., electrical resistive heating elements, cooling fans, refrigeration means, and any other suitable source of heating or cooling. Temperature control means also includes temperature sensors, e.g., a thermostat or thermocouple device, and means to turn the heating or cooling means on or off in response to detected temperature changes.

The apparatus also provides means to hold, move and manipulate one or more supports or cassettes to conduct ECL reactions. The apparatus may further comprise a stationary or moveable sample delivery means for placing a sample onto the PMAMS binding domains, as described for cassettes hereinabove.

The apparatus also comprises an electrode contact means able to electrically connect the array of separately addressable electrode connections of the cassette to an electronic voltage waveform generator means, e.g., potentiostat (see e.g., Fig. 5 of U.S. Patent 5,068,088). The waveform generator means delivers signals sequentially or simultaneously to independently trigger a plurality of ECL reactions in the cassette.

During an ECL assay, ionic current between working and counter electrodes may flow through ionically conducted liquid (for example water containing ionic salts), through a thin film of such liquid, and/or through an ionically conducting solid matrix.

Thus, an apparatus for measuring electrochemiluminescence in a sample can comprise a plurality of cells for holding at least one sample, wherein a cell may be formed from one or more electrodes and one or more counterelectrodes and a first support that comprises a plurality of discrete binding domains. The electrodes and counterelectrodes can be provided on the surface of the first support or on the surface of a second support wherein the second support is in close proximity to the binding domains on the first support. The electrodes and counterelectrodes may occur in pairs. The cell may further comprise a plurality of sensing electrodes to sense the voltage adjacent to the working electrode. The cassette may further comprise a cell containing a reference electrode.

The apparatus further comprises light detection means able to detect ECL reactions conducted in the cassette, e.g., by one or multiple detector means. Such detector means include, simply by way of example, an array of fiberoptic channels in register with the electrode array and positioned adjacent thereto, connected to an array of photodetector means, or to a single light detector means able to scan the array of ECL signals as emitted.

The apparatus optionally comprises a digital computer or microprocessor to control the functions of the various components of the apparatus.

The apparatus also comprises signal processing means. In one embodiment, and simply by way of example, the signal processing means comprises a digital computer for transferring, recording, analyzing and/or displaying the results of each ECL assay.

Alternatively, the apparatus comprises electrode translation means, for example, to scan one or more electrode/counterelectrode pairs across the binding surface to sequentially trigger ECL.

Size exclusion filters may be used in a parallel array of PMAMS.

5.10. ECL ASSAYS THAT MAY BE CONDUCTED

ECL labels for use according to the present invention can be selected from among ECL labels known in the art (see Section 2.2, above, and U.S. Patent No. 5,310,687). The ECL label may comprise, for example, a metal-containing organic compound wherein the metal is selected from the group consisting of ruthenium, osmium, rhenium, iridium, rhodium, platinum, palladium, molybdenum, technetium and tungsten. Suitable linking chemistry for preparing ECL TAG reagents is well known and disclosed, for example, by Bard et al. (U.S. Patent Nos. 5,310,687 and 5,221,605). The means of attachment of the ECL label to a binding reagent may be covalent and/or noncovalent. An ECL label may bind non-covalently to a binding reagent (e.g., through hydrophobic effects or ionic interactions). In other examples of non covalent attachment, ECL label(s) are bound (covalently or non-covalently) to a complex which in turn is non-covalently linked to a binding reagent. A more specific example would be covalent attachment of Ru(bpy)₃ through a linker to a Ni(II)-trinitrilotriacetic acid complex. This molecule will attach to binding reagents which include a peptide sequence containing a plurality of histidines. Other receptor ligand

sample. Alternatively, the sample may be contacted with the binding surface prior to contacting the binding surface with the ECL labeled ligand; the ECL labeled ligand will then compete with the previously bound analyte from the sample on the PMAMS surface and displace some of the previously bound analyte. In an alternative embodiment, the sample can be treated so as to contain substances/molecules that are ECL-labeled, and a standard amount of unlabeled analyte of interest can be contacted with the binding surface prior to or concurrently with contacting of the binding surface with the sample in order to carry out a competition assay.

In a sandwich assay, the ECL labeled ligand is a binding partner that specifically binds to a second binding moiety on the analyte of interest. Thus, when analyte that specifically binds to a binding reagent in a binding domain of a PMAMS is present in a sample, a "sandwich" is thus formed, consisting of the binding reagent on the binding domain, bound to analyte from the sample, bound to the ECL labeled binding partner. In another competitive sandwich assay, copies of the analyte itself are attached to the binding domains of the multi-array binding surface prior to exposure to the sample. Sample is then contacted with the binding surface. An ECL labeled binding partner (which can specifically bind to the analyte) will bind the analyte in the absence of free analyte (from sample) in the assay solution, but will be competitively inhibited in the presence of free analyte (from sample) in the assay solution.

In alternative embodiments, sequential labeling is performed. For example, in a particular embodiment of a sandwich assay, the analyte bound to the binding domain is contacted sequentially with a plurality of ECL labeled binding partners of the analyte. ECL measurements and optional washing steps are conducted in between contacting with each different binding partner. In this way an ECL measurement of a plurality of different binding moieties of an analyte may be performed (e.g., CD8⁺, a, b T cell antigen receptor positive T cell).

Additionally, multiple ECL labels, each emitting light at a distinguishable wavelength, may each be linked to a different binding reagent specific for a different moiety on an analyte. Further, a plurality of distinguishable reporter means (e.g., ECL label, fluorescent label and enzyme linked label) each attached to a different binding reagent specific for a different binding moiety of an analyte may be used, for example, to distinguish a CD4⁺, a, b T cell antigen receptor-positive cell from a CD8⁺ a, b T cell antigen receptor-positive cell.

In preferred embodiments the binding domains contain labelled proteins and/or nucleic acids and/or cells and/or chemical species. Such labelled components (e.g., ECL labels) may be added to the binding domain during fabrication, prior to the start of an assay, during an assay and/or at the end of an assay. For example, multiple labelled components may be added at various times and sequential readings may be taken. Such readings may provide cumulative information. In another embodiment, the binding domains of the PMAMS may be reused a multiplicity of times. After a given assay is performed, the surface may be washed under conditions which rejuvenates the activity of one or more binding domains of the PMAMS surface. By way of example, some binding reactions may be reversed by changing the ionic strength of the reaction solution. Alternatively, heat may be used to disassociate binding complexes. Some, binding domains may be inherently self-renewing. Binding domains which contain catalytic (e.g., enzymatic) functionalities may be utilized more than once. The binding domains are utilized continuously, and thus can be used in biosensor applications.

Additionally, the assay may be formatted so that the binding reagent attached to the multi-array multi-specific patterned surface is ECL labeled. Upon binding to certain analytes of interest in a sample, the ECL signal will be quantitatively modulated. For example, the ECL labeled binding reagent attached to the surface may be specific for an analyte on

a cell surface e.g., antigens such as alpha and beta T cell antigen receptor antigens, or CD4 or CD8 antigens. Upon exposure to a mixture of cells, cells bound to the surface will sterically hinder the ability of an electrode surface, brought into proximity with the multi-array multi-specific surface, from exciting the ECL labeled binding reagent thus down-modulating the ECL signal.

Homogeneous and heterogenous assays may be conducted. In heterogeneous assays, unbound labeled reagent is separated from bound labeled reagent (e.g., by a washing step) prior to exposure of the bound or unbound labeled reagent to an electrical potential. In homogeneous assays, unbound labeled reagent and bound labeled reagent are exposed to an electrical potential together. In homogeneous assays, the intensity or spectral characteristics of the signal emitted by the bound labeled reagent is either greater than or less than the intensity of the signal emitted by the unbound labeled reagent. The presence or absence of the respective bound and unbound components can be determined by measuring the difference in intensity.

Once the desired steps of contacting the binding reagents with analyte or competitor thereof and any binding partners thereto, have been completed, one then ensures that the ECL label is subjected to an environment conducive to ECL. Suitable ECL assay medium are known in the art. Such an assay medium advantageously includes a molecule that promotes ECL of an ECL label, including but not limited to oxalate, NADH, and most preferably tripropylamine. Such a "promoter" molecule can be provided free in solution, or can be provided by prior linkage to or by production at (e.g., as a product of a chemical reaction) the PMAMS surface, a monolayer on the surface, the binding domain, the electrode surface, a binding reagent, and/or an ECL label, etc. If the medium surrounding the ECL label bound to the binding domains resulting from the contacting steps is conducive to ECL, no changes to the medium need be made. Alternatively, the medium can be adjusted or replaced to provide a medium

conducive to ECL. An electrode and counterelectrode is already proximate to the binding domain, or is brought near or in contact with the binding domain, a voltage waveform is applied, and ECL is detected or measured.

In a preferred embodiment of the invention, the above-described steps of contacting the binding reagents with analyte or competitor thereof and any binding partners thereto, are carried out in the absence of electrodes and counterelectrodes, i.e., such that the sample does not contact the electrode or counterelectrode. Subsequent to these contacting steps, electrodes and counterelectrodes are brought sufficiently close to the ECL label bound to the binding domain, to trigger an ECL reaction.

A support having a PMAMS may be used for sequencing of nucleic acid strands. For example, a PMAMS with a plurality of binding domains is prepared with different oligonucleotide probes of known nucleotide sequence as the binding reagents in different binding domains. That is, different binding domains will contain binding reagents of different known nucleotide sequence. The oligonucleotide chain or fragments of the oligonucleotide chain to be sequenced are then allowed to bind (hybridize) to the PMAMS binding domains. The nucleic acids to be sequenced are ECL labeled. Binding assays are conducted on the PMAMS and the distribution of ECL signals from the discrete binding domains on the PMAMS is used to sequence the oligonucleotide chain.

The above-described method is based on the ability of short oligonucleotides to hybridize to their complementary or substantially complementary sequence in another nucleic acid molecule (see, e.g., Strezoska et al., 1991, *Proc. Natl. Acad. Sci. USA* 88: 1089-1093; Bains, 1992, *Bio/Technology* 10: 757-58, which are incorporated herein by reference). Conditions can be selected such that the desired degree of sequence complementarity is necessary for successful hybridization. Hybridization of a DNA molecule of unknown sequence to a probe of predetermined sequence detects the presence of the complementary sequence in

the DNA molecule. The method is preferably practiced such that the hybridization reaction is carried out with the oligonucleotide probes bound to the binding domains and the sample DNA in solution.

A PMAMS may also be utilized to isolate, screen and/or select a novel molecule or complex of desired function (e.g. binding or catalysis). A PMAMS may be used to isolate compounds and/or lead compounds for therapeutic uses. A PMAMS containing a plurality of peptides, nucleic acids, viral vectors, or polymers, synthesized by a variety of combinatorial chemistries, can be made using the methods of the invention. A wide variety of such PMAMS treated supports may be used to rapidly screen for binding to, for example, an ECL labeled cellular receptor. In one method a first PMAMS with a large diversity of unrelated peptide sequences is used in order to isolate lead binding peptide sequences. Then a PMAMS with peptides of related sequences to those which showed binding to the molecule of interest (e.g., a cellular receptor) on the first PMAMS are then used. The process is repeated until a peptide with the desired binding characteristics are found.

An analyte of interest may be, e.g., a whole cell, a subcellular particle, virus, prion, viroid, nucleic acid, protein, antigen, lipoprotein, lipopolysaccharide, lipid, glycoprotein, carbohydrate moiety, cellulose derivative, antibody or fragment thereof, peptide, hormone, pharmacological agent, cell or cellular components, organic compounds, non-biological polymer, synthetic organic molecule, organo-metallic compounds or an inorganic molecule present in the sample.

The sample may be derived from, for example, a solid, emulsion, suspension, liquid or gas. Furthermore, the sample may be derived from, for example, body fluids or tissues, water, food, blood, serum, plasma, urine, feces, tissue, saliva, oils, organic solvents or air. The sample may comprise a reducing agent or an oxidizing agent.

Assays to detect or measure the following substances may be conducted by the present invention, by incorporation of a binding reagent specific to said substances into the binding domains of the binding surfaces of the invention: albumin, alkaline phosphatase, alt/SGPT, ammonia, amylase, AST/SGOT, bilirubin-total, blood urea nitrogen, calcium, carbon dioxide, chloride, cholesterol-total, creatinine, GGT, glucose, HDL cholesterol, iron, LDH, magnesium, phosphorus, potassium, protein-total, sodium, triglycerides, uric acid, drugs of abuse, hormones, cardiovascular system modulators, tumor markers, infectious disease antigens, allergy provoking antigens, immunoproteins, cytokines anemia/metabolic markers, carbamazepine, digoxin, gentamicin, lithium, phenobarbital, phenytoin, procainamide, quinidine, theophylline, tobramycin, valproic acid, vancomycin, amphetamines, antidepressants, barbiturates, benzodiazepines, cannabinoids, cocaine, LSD, methadone, methaqualone, opiates, phenylethylamine, propoxyphene, ethanol, salicylate, acetaminophen, estradiol, progesterone, testosterone, hCG/bhCG, follicle stimulating hormone, luteinizing hormone, prolactin, thyroid hormones such as thyroid stimulating hormone, T4, TUP, total-T3, free-T4, free-T3, cortisol, creatinine kinase-MB, total-creatinine kinase, PT, APTT/PTT, LD ISOs, creatinine kinase ISOs, myoglobin, myo light chain, troponin I, troponin T, chlamydia, gonorrhea, herpes virus, Lyme disease, Epstein Barr virus, IgE, Rubella-G, Rubella-M, CMV-G, CMV-M, toxo-G, toxo-M, HBsAg (hepatitis B virus surface antigen), HIV 1, HIV 2, anti-HBc, anti-HBs, HCV, anti-HAV IgM, anti-HBc IgM, anti-HAV, HBeAg, anti-HBeAg, TB, prostate specific antigen, CEA, AFP, PAP, CA125, CA15-3, CA19-9, b2-microglobulin, hemoglobin, red blood cells, HBcAb, HTLV, ALT, STS-syphilis, ABO blood type antigens and other blood typing antigens, cytomegalovirus, ferritin, B-12, folate, glycosylated hemoglobin, amphetamines, antidepressants and other psychotropic pharmaceuticals.

Measurements of ECL at different binding domains can be done sequentially or simultaneously.

A PMAMS specific for an analyte of interest that is a cell-surface protein is first exposed to a sample containing cells, in which it is desired to count the cells in the sample. In a preferred embodiment, a known sample volume and/or diluted sample is exposed to a PMAMS which has a multiplicity of binding domains specific for at least one cell surface antigen. Bound cells can then be quantified by attachment of a secondary binding group linked to an ECL tag. This is a group capable of interacting with a broad range of cell types, for example an ECL-Tag linked to a hydrophobic group capable of inserting into a cell membrane or to a lectin directed against cell surface sugars. The ECL-Tag is linked to a secondary antibody directed against a cell surface antibody. In a more specific embodiment, several cell types bound to the same domain can be distinguished by the use of multiple ECL-Tag labeled secondary antibodies. One preferably ensures that the number of discrete binding domains specific for a given analyte on the surface of a cell exceeds the average number of cells that will bind that are present in the sample. Statistical techniques can then be utilized to determine the number of cells per sample volume. This technique can also be used, e.g., to count other particles such as viruses, where the binding reagent recognizes an antigen on the virus. The domains can be small compared to the size of a cell so that only one cell can bind per domain, thus leading to a digital signal for each domain which can then be analyzed over the sum of the domains using statistical methods. The domains are large compared to the size of a cell so that multiple cells can bind to a domain. In this case, the level of signal from each domain can be calibrated to give the number of cells per volume of sample. Image analysis using an array of light detectors (e.g., a CCD camera or avalanche photodiode array) could be used to count cells and determine cell morphologies.

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The invention preferably also provides for methods for conducting ECL reactions, e.g., assays, at a rate of to 1000 ECL reactions in from 5 to 15 minutes.

5.11. PMAMS FOR USE WITH OTHER
ANALYTIC METHODS AND/OR ECL

The techniques described above for ECL based detection can be used in conjunction with other assay techniques, e.g., as domains in which catalyses and other chemical reactions can occur. Discrete binding domains according to the invention may be used in other assay techniques such as, clinical chemical chemistry assays, e.g., electrolyte determinations, clinical enzyme determinations, blood protein determinations, glucose, urea and creatinine determinations, and the like. Other assay techniques that may be combined with ECL assays and/or used alone with the PMAMS of the invention include chemiluminescent based label, fluorescent based assays, enzyme-linked assay systems, electrochemical assays (see, e.g., Hickman et al., 1991, Science 252:688-691) and/or resonance detection (e.g., surface plasmon and acoustic techniques) assay systems.

PMAS supports with drops may be utilized in which there is a plurality of different chemistries within the array of drops. Each drop may contain different binding reagents and/or different chemical assays (i.e., reaction medium for the same). For example, the drops may be hydrophilic, resting on hydrophilic surface binding domains which are surrounded by hydrophobic surface regions. The drops are protected by a hydrophobic solution covering the surface. The hydrophilic solution to be assayed is deposited on a second PMAMS with hydrophilic binding domains surrounded by hydrophobic regions. The two surfaces are brought into registered proximity so as to bring into contact the hydrophilic domains on the opposite surfaces and a spectral analysis is performed to detect reaction products of the chemical assays.

The fibril mats may be patterned such that there are a plurality of discrete hydrophobic and/or hydrophilic domains surrounded by hydrophilic and/or hydrophobic domains. Drops of

aqueous solutions containing binding reagents may rest on hydrophilic regions and be confined by surrounding hydrophobic regions. These drops may contain, for example, fibrils, aggregates of fibrils, binding reagents, ECL reagents, reagents for assays, surfactants, PEGs, detergents, a plurality of biological molecules mentioned above by example, and/or combinations thereof.

The hydrophobic solution covering the first PMAMS is controllably removed (e.g., evaporated, wicked) so as to expose only a portion of the hydrophilic drops at the tops to the environment. A hydrophilic solution to be assayed for an optical chemical reaction is then exposed to the PMAMS surface--the hydrophilic micro-drops and the solution to be assayed mix and analysis (e.g., spectral) is performed.

PMAMS binding domains may also be used as a pre-filter or filter. For instance, a cellular specific PMAMS can be used in certain instances alone as a filter for certain cell types as well as in conjunction with a size exclusion filter. The resulting analyte solution is then exposed to a PMAMS specific for subcellular particulate matter (e.g., viruses). The particulate subcellular PMAMS and/or a size exclusion filter is used to generate a small molecule (e.g., protein, small chemical entities) analyte solution. By utilizing a serial PMAMS assay system the analyte solution may be sequentially purified in order to decrease non-specific analyte interactions.

The optical opacity of a material used for a support, electrode and/or binding domain may be varied to achieve desired properties. Such a material may be translucent, transparent or substantially opaque, depending on the thickness, compositing and/or optical density of the material.

The optical opacity of fibril mats increases with increasing thickness of the mat. Very thin mats are optically translucent. Thicker mats can be substantially opaque. In some examples, mats that range in thickness from 0.01 μm to 0.5 μm are substantially translucent. In other examples, mats with a

thickness greater than 20 μm are substantially opaque. Mats with a thickness between 0.5 μm and 20 μm have intermediate opacity, which increases with increasing thickness of the fibril mat. The optical opacity of a particular thickness of a mat may depend on the composition, density, derivatization, number of layers, types and quantities of materials dispersed in the mat, and/or a combination thereof. It may also depend on the wavelength of the light used.

If a material is substantially translucent at a given thickness and substantially opaque for another thickness, light emitted from a certain depth in the material may pass out of the material while light emitted from another (e.g. greater) depth may be substantially absorbed or scattered by the material. In one example, the variable opacity of a material allows the material to be used as an optical filter.

Light emitted from a certain depth in a fibril mat may pass substantially through the mat and be observed with a detector placed on or in proximity to a surface of the fibril mat. Light emitted from another depth may be substantially absorbed and/or scattered by the mat and not be observed by a detector placed on or in proximity to the surface of the mat. This property of a fibril mat (and/or optically similar materials) may be used to distinguish between bound and unbound reagents in ECL assay.

Certain reagents can diffuse (actively or passively), be pulled (e.g., by suction filtration and/or by capillary action), wicked, or pushed by pressure to a sufficient depth in a porous material that emission of light from these reagents is substantially or entirely absorbed or scattered by the mat. In one example, a fibril mat acts as both a physical and an optical filter through which certain reagents are passed, certain reagents are entrained, and/or certain reagents bind to a very thin layer either at or in proximity to the surface of the mat. Reagent bound to one or more binding domains and/or species bound to species bound to one or more binding domains (these domains being

located either on the surface of the fibril mat or in a very thin layer near the surface of the mat on the PMAMS) are prevented from diffusing, being pulled, etc. into or through the mat. Reagents and/or other solutions are flowed or suspended on and/or over the surface of the fibril mat such that reagents bind only to a very thin layer on the surface of the mat. Reagents can be washed through the mats, once or many times, in one or more directions. Reagents may bind to the fibril mat, one or more binding domains, other or the same reagents bound to one or more binding domains, be entrained inside the mat, pass through the mat, or a combination thereof.

Porous materials used in supports and/or electrodes may have more than one layer in which the upper layer has binding domains and other layers within the mat do not have binding domains. In one example, a fibril mat, (illustrated schematically in Fig. 29), the upper layer 2900 is thick enough to prevent passage of light that originates in layer(s) 2901, 2902 from the mat below this layer. Light 2903 that originates from sources 2904, 2905 bound to this upper layer can be detected by a detector 2906 located at or in proximity to the surface of the mat. Light originating from sources 2907, 2908, 2909 in lower layers 2901, 2902 is absorbed and/or scattered by either or all layers and cannot be detected by the detectors 2906, 2910.

A pre-filtration step may be used to select particular sizes, types, derivatives of fibrils and/or fibril aggregates before the mat is fabricated. The filter agent used to filter a suspension of fibrils is a mat of fibrils of a certain or many porosities.

A porous material (e.g. a fibril mat) may act as the support for the binding domains, an electrode which may be used for ECL or other electrochemical applications, a filter that can be used to control delivery of reagents, and/or an optical filter that can transmit, absorb and/or scatter light to varying degrees.

5.12. ELECTROCHROMIC ECL DISPLAY PANELS

droplets described in the two embodiments above are stabilized using hydrophobic solutions. The viscosity of the solutions may be increased to increase the stability of the droplet arrays. The hydrophilicity and hydrophobicity may be reversed. In other embodiments the droplets may contain solutions capable of polymerizing to increase the stability and/or conductivity (e.g., conducting polymers) of the film between or above the electrode pairs. Additionally, structural features may be utilized to limit cross-talk between pixels. For example, an elastomeric stamp (e.g., poly(dimethylsiloxane)) with ring shaped stamp protrusion features capable of circumscribing side by side electrode pixel pairs on a surface may be used to isolate electrolytic solutions, gels, or films between pixels. Alternatively, side by side electrode pixel pairs may be placed in electrically insulating well-like structures on a surface, electrolytic solutions, gels or films placed in the wells above the electrodes, and the entire surface covered or coated to isolate and contain the electrolytic components of each pixel.

5.13. PMAMS FOR USE IN OTHER CHEMICAL REACTIONS

The PMAMS of the invention can also be used to conduct chemical reactions not in combination with ECL. For example, all the techniques and non-ECL assays discussed in Section 5.11 above can be used.

A cassette is provided for detecting or measuring an analyte of interest in a sample, comprising: (a) a first support having a plurality of discrete binding domains on the surface thereof to form at least one binding surface, at least some of the discrete binding domains being of different binding specificities than other binding domains, each of the plurality of discrete binding domains being hydrophilic and surrounded by hydrophobic regions, and (b) a second support having a plurality of hydrophilic domains comprising reaction media suitable for conducting a chemical assay thereon to form an assay surface, in which the plurality of discrete binding domains and the plurality of reaction media is capable of being brought into contact so

that a sample to be analyzed present on each binding domain is contacted with a reaction medium to detect or measure an analyte of interest. Alternatively, the binding domains can be hydrophobic, and the second support has a plurality of hydrophobic domains containing reaction medium.

The invention also provides a method for detecting or measuring analytes of interest in a sample, comprising: (a) placing drops of a sample containing an analyte to be detected or measured on a plurality of discrete binding domains on a support surface, in which the plurality of discrete binding domains comprises at least one binding domain that contains binding reagents that are identical to each other and that differ in specificity from the binding reagents contained within other binding domains, each of the discrete binding domains being characterized as either hydrophobic or hydrophilic, with the proviso that the region of the support surface surrounding each binding domain is (i) hydrophobic if the binding domain is hydrophilic, and (ii) hydrophilic if the binding domain is hydrophobic, so as to allow one or more analytes of interest in the sample to bind to the binding domains, and (b) contacting the drops on the first support with a surface of a second support having a plurality of discrete hydrophilic domains comprising reaction media suitable for conducting a chemical assay thereon, and (c) determining the presence of the analytes of interest that are bound to the binding domain.

Also provided is a method for detecting or measuring analytes of interest in a sample, comprising (a) placing drops of a sample containing an analyte to be detected or measured on a plurality of discrete binding domains on a support surface in which the plurality of discrete binding domains comprises at least one binding domain that contains binding reagents that are identical to each other and that differ in specificity from the binding reagents contained within other binding domains, each of the discrete binding domains being characterized as either hydrophobic or hydrophilic, with the proviso that the region of

the support surface surrounding each of the binding domains is (i) hydrophobic if the binding domain is hydrophilic, and (ii) hydrophilic if the binding domain is hydrophobic, so as to allow one or more analytes of interest in the sample to bind to the binding domains, and (b) placing drops of a reaction medium on the drops of sample; and (c) determining the presence of analytes of interest that are bound to the binding domain.

In a particular example of this aspect of the invention, binding domains, each of which have incorporated a different enzyme that utilizes as a substrate a sequential intermediate in a chemical reaction are situated on a PMAMS surface, such that the product of a given enzymatic reaction, which is the reactant for a subsequent enzyme, flows to the next enzyme in the reaction pathway. The invention also provides for bulk immobilization of enzymes on self-assembling monolayers, e.g., for industrial application, using methods as described above.

For example, sheets with such immobilized enzymes on one or both sides may be stacked to achieve high surface area to solution volume ratios. Alternatively, such immobilized enzymes may be attached to porous materials. Additionally, such immobilized enzymes may be on dipsticks, stirring agents, on the walls of tubes or capillaries, or on the walls of containers such as an incubator chamber.

In an alternative aspect of the invention, non-ECL assays such as described above can be carried out on PMAMS analogs, said PMAMS analogs differing from PMAMS as described above in that the PMAMS analogs contain discrete domains for carrying out non-ECL reactions, the discrete domains not necessarily having incorporated a binding reagent and therefore not necessarily being binding domains. Such PMAMS analogs have discrete domains for carrying out reactions and are prepared so as to inhibit spreading and/or diffusion of fluid applied to the discrete domains. In one embodiment, the domains are either hydrophobic or hydrophilic relative to the surrounding regions on

the support surface, in order to aid in confining the reaction medium and/or sample to the discrete domains. The use of wells, deposition of reaction medium or sample on felts or porous materials, deposition and drying of reaction medium or sample on gels, films, etc., can be used to inhibit spreading or diffusion. Each of such discrete domains is less than 1 mm in diameter or width, preferably in the range of 50 nm to 1 mm, most preferably in the range of 1 micron to 1 mm. The same or different reaction medium can be deposited on each of the discrete domains prior to sample application, or sample application can precede deposition of reaction medium.

In a preferred aspect of the use of PMAMS analogs to conduct non-ECL assays, drops of reaction medium are placed on a plurality of discrete domains, preferably delivered concurrently from an array of microfluidic guides; and then, optionally, to enhance stability and/or protect the drop, a more viscous solution (e.g., oil) is placed on top of the reaction medium or, alternatively, in between the discrete domain; and then sample containing an analyte to be detected or measured is applied to each domain, either by discrete application to each discrete domain or, in bulk, by exposing the entire surface of the PMAMS analog containing the domains to a fluid sample. Any resulting reaction in the binding domains is allowed to proceed, and the results are observed by use of a reporter and detection system selected from among those known in the art.

The invention is further described in the following examples which are in no way intended to limit the scope of the invention.

6. EXAMPLES

6.1. PREPARATION OF AN MAB PMAMS SURFACE BY MICRO-STAMPING

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures in a square array pattern. A 10:1 mixture of SYLGARD silicone elastomer 184 (poly(dimethylsiloxane); available from Dow Corning) and the corresponding SYLGARD 184 curing agent is

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poured over the master and cured. The polymerized SYLGARD 184 is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a hydrophilic OH-terminated alkane thiol, $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$, in an ethanolic solution (1-10 mM), robotically brought into pin registered contact with an aligned gold surface, and removed. The substrate is then washed for a few seconds (e.g., 2-10 seconds) with a solution of a hydrophobic CH_3 -terminated alkane thiol, $\text{SH}(\text{CH}_2)_{10}\text{CH}_3$ (1-10 mM in ethanol) (Kumar et al., *supra* and Prime et al., Science 252:1164-7). The resulting surface is then gently dried under a stream of nitrogen. A capillary array containing hydrophilic solutions is then robotically brought into pin registered contact with the aligned surface aligning the capillaries with the $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$ domains. Each capillary in the capillary array contains monoclonal antibodies (MABs), specific for an analyte of interest, capable of covalently binding to the reactive OH groups on the hydrophilic domains through an amide linkage.

6.2. PREPARATION OF AN MAB AND NUCLEIC ACID PMAMS SURFACE BY MICRO-STAMPING

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures in a square array pattern. A 10:1 mixture of SYLGARD silicone elastomer 184 and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD 184 is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a hydrophilic OH-terminated alkane thiol, $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$, in an ethanolic solution (1-10 mM), robotically brought into pin registered contact with an aligned gold surface, and removed. The substrate is then washed for a few seconds (e.g., 2-10 seconds) with a solution of a hydrophobic CH_3 -terminated alkane thiol, $\text{SH}(\text{CH}_2)_{10}\text{CH}_3$ (1-10 mM in ethanol) (Kumar et al., *supra* and Prime et al., Science 252:1164-7). The resulting surface is then gently dried under a stream of nitrogen. A capillary array containing hydrophilic solutions is then robotically brought into

pin registered contact with the aligned surface aligning the capillaries with the $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$ domains. Each capillary in the capillary array contains antibodies or modified nucleic acids, specific for an analyte of interest, capable of covalently binding to the reactive OH groups on the hydrophilic domains through amide bond linkages.

6.3. PREPARATION OF A PMAMS SURFACE BY ETCHING

A clean gold surface is exposed to a hydrophilic OH-terminated alkane thiol, $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$ (Prime et al., Science 252:1164-1167) in an ethanolic solution (1-10 mM). A linear array of fine tipped etching utensils is robotically brought into optically registered contact with an aligned gold surface, and the linear array is used to etch in both the X and Y dimensions of the surface creating a two dimensional grid array of $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$ domains. The substrate is then washed for a few seconds (eg. 2-10 seconds) with a solution of a hydrophobic $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{CH}_3$ (1-10 mM in ethanol). The resulting surface is then gently dried under a stream of nitrogen. A capillary array containing hydrophilic solutions is then robotically brought into pin registered contact with the surface aligning the capillaries with the $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$ domains. Each capillary in the capillary array contains antibodies or nucleic acids, specific for an analyte of interest, capable of covalently binding to the reactive OH groups on the hydrophilic domains.

6.4. SANDWICH ASSAY ON A PMAMS SURFACE

A transparent PMAMS surface is made as described above which is substantially transparent with a patterned multi-specific array of primary antibodies linked to the surface. The support, electrode assay, monologues surface use selected to be transparent. The PMAMS surface is then exposed to a solution sample suspected of containing an analyte of interest to be assayed. The sample is then washed away leaving antibody bound analytes on the surface. The PMAMS surface is then exposed to a solution containing secondary ECL-labeled antibodies specific for

bound analytes on the surface. This solution is then washed from the PMAMS surface leaving ECL labeled secondary antibodies bound to the domains where analyte is present.

The electrode assay is protected by a removable barrier to prevent premature contact of the sample with the electrode surface in order to avoid contamination effects. The barrier is then removed and the electrode array, that is wetted with assay buffer, is brought into aligned contact with the PMAMS surface. The electrode array is connected to an electronic potential wave form generator, and potential is applied to working electrode/counter electrode pairs. A CCD then reads the light emitted and the signal is sent to a microprocessor which converts the signal to the desired readout form.

The readout is compared to the readout obtained using controls in the form of known quantities of an analyte of interest to calculate the actual quantity of analyte.

6.5. ASSAY ON A FIRST AND SECOND PMAMS SURFACE

A transparent PMAMS surface is made as described above with a patterned multi-specific array of primary antibodies linked to the surface. The PMAMS surface is then exposed to a solution sample suspected of containing an analyte of interest to be assayed. The sample is then washed away leaving antibody bound analytes on the surface.

A second PMAMS, under a protective cover, is provided, with an alternating hydrophobic/hydrophilic pattern on which there are patterned micro-drops of a plurality of secondary antibodies labeled with ECL tag.

The barrier protecting the second PMAMS in register with the first PMAMS is removed and the micro-drops are brought into register with the primary antibody binding domains on the first PMAMS. The second PMAMS is lifted off and the electrode array and is brought into aligned contact with the first PMAMS surface. The electrode array is connected to an electrical potential wave form generator, and potential is applied to working electrode/counter electrode pairs. A photo multiplier

tube then reads the light emitted and the signal is sent to a microprocessor which converts the signal to the desired readout form.

The readout is compared to the readout obtained using controls in the form of known quantities of an analyte of interest to calculate the actual quantity of analyte.

6.6. NUCLEIC ACID ASSAY ON A PMAMS SURFACE

A transparent PMAMS surface is made as described above with a patterned multi-specific array of single-stranded nucleic acid probes linked to the surface. The probes are complementary to the 5' region of a nucleic acid analyte of interest. The PMAMS surface is then exposed to a solution sample suspected of containing a hybridizable nucleic acid analyte of interest to be assayed, the sample having been previously denatured, i.e., treated to render the analyte of interest single stranded. The sample is then washed away leaving hybridized analytes on the surface. The PMAMS surface is then exposed to a solution containing secondary ECL labeled nucleic acid probes specific for the 3' terminus of the nucleic acid analytes bound on the surface. This solution is then washed from the PMAMS surface leaving ECL labeled nucleic acid probes bound to the domains where analyte is present.

The barrier protecting the second PMAMS in register with the first PMAMS is removed and the micro-drops are brought into register with the primary antibody binding domains on the first PMAMS. The second PMAMS is lifted off and the electrode array and is brought into aligned contact with the first PMAMS surface.

The electrode array is connected to an electronic potential wave form generator, and potential is applied to working electrode/counterelectrode pairs. A CCD then reads the light emitted and the signal is sent to a microprocessor which converts the signal to the desired readout form.

The readout is compared to the readout obtained using controls in the form of known quantities of an analyte of interest to calculate the actual quantity of analyte.

6.7. COMPETITIVE ASSAY ON A PMAMS SURFACE WITH A PHOTOMULTIPLIER DETECTOR

A transparent PMAMS surface is made as described above with a patterned multi-specific array of primary antibodies, specific for an analyte of interest, linked to the surface. The PMAMS surface is then exposed to a solution sample to be assayed which is a mixture of a sample suspected of containing the analyte of interest and a known amount of an ECL labeled molecule competitive with the analyte of interest for binding to the antibodies. The sample is then washed away leaving antibody bound analytes and/or labelled competitive binders on the surface.

The electrode array is protected by a removable barrier to prevent contact of the sample with the electrode surface in order to avoid contamination effects. The barrier is then removed and the electrode array, that is wetted with assay buffer, is brought into aligned contact with the PMAMS surface. The electrode array is connected to a electronic potential wave form generator, and potential is applied to working electrode/counter electrode pairs. A photomultiplier tube then reads the light emitted and the signal is sent to a microprocessor which converts the signal to the desired readout form.

The readout is compared to the readout obtained using controls in the form of known quantities of an analyte of interest to calculate the actual quantity of analyte.

6.8. COMPETITIVE ASSAY ON A PMAMS SURFACE WITH A CCD DETECTOR

A transparent PMAMS surface is made as described above with a patterned multi-specific array of primary antibodies linked to the surface. The PMAMS surface is then exposed to a solution sample suspected of containing the analyte of interest

to be assayed. The sample is then washed away leaving antibody bound analytes on the surface.

A second PMAMS, under a protective cover, is provided with an alternating hydrophobic/hydrophilic pattern on which there are patterned micro-drops of a plurality of a known amount of an ECL labeled molecule competitive with an analyte of interest.

The barrier protecting the second PMAMS in register with the first PMAMS is removed and the micro-drops are brought into register with the primary antibody binding domains on the first PMAMS. The second PMAMS is lifted off and the electrode array is brought into aligned contact with the PMAMS surface. The electrode array is connected to a electronic potential wave form generator, and potential is applied to working electrode/counter electrode pairs. A CCD then reads the light emitted and the signal is sent to a microprocessor which converts the signal to the desired readout form.

The readout is compared to the readout obtained using controls in the form of known quantities of an analyte of interest to calculate the actual quantity of analyte.

6.9. PREPARATION OF AN MAB PMAMS SURFACE BY MICRO STAMPING WITH AN $\text{SH}(\text{CH}_2)_{10}\text{CH}_3$ ALKANE THIOL

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures in a square array pattern. A 10:1 mixture of SYLGARD silicone elastomer 184 (poly(dimethylsiloxane); available from Dow Corning) and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD 184 is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a hydrophilic OH-terminated alkane thiol, $\text{SH}(\text{CH}_2)_{11}\text{OH}$, in an ethanolic solution (1-10 mM), robotically brought into pin registered contact with an aligned gold surface, and removed. The substrate is then washed for a few seconds (e.g., 2-10 seconds) with a solution of a hydrophobic CH_3 -terminated alkane thiol, $\text{SH}(\text{CH}_2)_{10}\text{CH}_3$ (1-10 mM in ethanol) (Kumar et al., *supra*). The resulting surface is

then gently dried under a stream of nitrogen. A capillary array containing hydrophilic solutions is then robotically brought into pin registered contact with the aligned surface aligning the capillaries with the $\text{SH}(\text{CH}_2)_{11}\text{OH}$ domains to place specific antibodies at each domain. Each capillary in the capillary array contains monoclonal antibodies, specific for an analyte of interest, capable of covalently binding to the reactive OH groups on the hydrophilic domains.

6.10. PREPARATION OF AN MAB and NUCLEIC
ACID PMAMS SURFACE BY MICRO-STAMPING
WITH AN $\text{SH}(\text{CH}_2)_{10}\text{CH}_3$ ALKANE THIOL

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures in a square array pattern. A 10:1 mixture of SYLGARD silicone elastomer 184 and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD 184 is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a hydrophilic OH-terminated alkane thiol, $\text{SH}(\text{CH}_2)_{11}\text{OH}$, in an ethanolic solution (1-10 mM), robotically brought into pin registered contact with an aligned gold surface, and removed. The substrate is then washed for a few seconds (e.g., 2-10 seconds) with a solution of a hydrophobic CH_3 -terminated alkane thiol, $\text{SH}(\text{CH}_2)_{10}\text{CH}_3$ (1-10 Mm in ethanol) (Kumar et al., *supra*). The resulting surface is then gently dried under a stream of nitrogen. A capillary array containing hydrophilic solutions is then robotically brought into pin registered contact with the aligned surface aligning the capillaries with the $\text{SH}(\text{CH}_2)_{11}\text{OH}$, domains to place specific antibodies and/or hybridizable nucleic acids at each domain. Each capillary in the capillary array contains antibodies or modified nucleic acids, specific for an analyte of interest, capable of covalently binding to the reactive OH groups on the hydrophilic domains through amide bond linkages.

6.11. PREPARATION OF A PMAMS SURFACE
USING A STREPTAVIDIN-BIOTIN LINKER

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An exposed and developed photoresist master of 1-2mm thickness is prepared according to well known procedures in a square array pattern. A 10:1 mixture of SYLGARD silicone elastomer 184 and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD 184 is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a mixture of mercaptoundecanol and 12-mercapto(8-biotinamide-3,6-dioxaoctyl)dodecanamide where the mole fraction of the biotinylated thiol is 0.1 (see Spinke et al., 1993, Langmuir 9:1821-5 and Spinke et al., 1993, J. Chem. Phys. 99(9): 7012-9). The substrate is then washed for a few seconds (e.g., 2-10 seconds) with a solution of a hydrophobic CH₃-terminated alkane thiol, HS(CH₂)₁₀CH₃ alkane thiol (1-10 mM in ethanol) (see Kumar et al. *supra*, Biebuyck, Whitesides). The resulting surface is then gently dried under a stream of nitrogen. A capillary array containing a solution of streptavidin in each capillary is then robotically brought into pin registered contact with the aligned surface. Each capillary in the capillary array is aligned and brought into contact with a biotinylated domain and the capillary array is removed and the surface washed. A second capillary array containing a multiplicity of biotinylated antibodies and biotinylated nucleic acids solutions is then robotically brought into pin registered contact with the aligned surface to place specific antibodies and nucleic acids on each domain.

6.12. PREPARATION OF AN MAB SINGLE SURFACE

An electrode array of interdigitating working and counterelectrode pairs on a gold on silicon surface is fabricated through methods known in the art (for example see Kumar et al *supra*). In this example, the electrode array and the discrete binding domain array exist on the same surface of a support. An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures in the pattern of the working electrodes. A 10:1 mixture of SYLGARD silicone elastomer 184 (poly(dimethylsiloxane(PDMS))); available from Dow

Corning) and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD 184 is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a hydrophilic OH-terminated alkane thiol, $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$, in an ethanolic solution (1-10 mM), robotically brought into pin registered contact with the aligned working electrodes on gold electrode array surface, and is then removed. A capillary array containing hydrophilic solutions is then robotically brought into pin registered contact by aligning the capillaries with the $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$ domains on the electrode array surface to place specific antibodies on each domain. Each capillary in the capillary array contains monoclonal antibodies, specific for an analyte of interest, capable of covalently binding to the reactive OH groups on the hydrophilic domains through an amide linkage.

6.13. ASSAY CONDUCTED ON AN MAB SINGLE SURFACE

A support as described 6.12, *supra*, is fabricated. A PDMS stamp is fabricated as previously described from a photoresist master patterned as rings which each independently circumscribe a working electrode/counterelectrode pair. The electrode array surface is then exposed to a sample to be analyzed, washed with a mixture of ECL labelled secondary antibodies, and then washed with an assay buffer solution containing tripropyl amine. The PDMS stamp is then aligned and brought into registered contact aligning the rings of the PDMS stamp so as to circumscribe and define individual volume elements of assay buffer above each electrode pair. An overpotential is applied to the electrode pairs so as to release the monolayer from the surface exposing the working electrode to the ECL labelled secondary antibodies. A photomultiplier tube then reads the light emitted through the transparent PDMS and the signal is sent to a microprocessor which converts the signal to the desired readout form.

The readout is compared to the readout obtained using controls in the form of known quantities of an analyte of interest to calculate the actual quantity of analyte.

6.14. PREPARATION OF A SINGLE SURFACE WITH WORKING AND COUNTERELECTRODES

An electrode array of interdigitating working and counter gold electrode pairs with gold binding domains in between the interdigitating electrodes on a gold on silicon support is fabricated through methods known in the art (for example see Kumar et al. *supra*). In this example, the electrode array and the discrete binding domain array exist on the same surface. An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures in the pattern of the binding domains in between the interdigitating electrode pairs. A 10:1 mixture of SYLGARD silicone elastomer 184 (poly(dimethylsiloxane(PDMS)); available from Dow Corning) and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD 184 is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a hydrophilic OH-terminated alkane thiol, $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$, in an ethanolic solution (1-10 mM), robotically brought into pin registered contact with the aligned gold binding domains on the electrode array surface, and is then removed. A capillary array containing hydrophilic solutions is then robotically brought into pin registered contact, aligning the capillaries with the $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$ domains on the electrode array surface to place specific antibodies on each domain. Each capillary in the capillary array contains antibodies specific for an analyte of interest, capable of covalently binding to the reactive OH groups on the hydrophilic domains through an amide linkage.

6.15. ASSAY CONDUCTED ON A SINGLE SURFACE WITH WORKING AND COUNTERELECTRODES

A support surface as described 6.14 *supra* is fabricated by the described methods. The prepared surface is exposed to a sample to be analyzed, washed with a mixture of ECL labelled

secondary antibodies, and then washed with an assay buffer solution containing tripropyl amine. The electrode array is connected to a electronic potential wave form generator, and potential is applied to working electrode/counterelectrode pairs. A photomultiplier tube then reads the light emitted and the signal is sent to a microprocessor which converts the signal to the desired readout form.

The readout is compared to the readout obtained using controls in the form of known quantities of an analyte of interest to calculate the actual quantity of analyte.

6.16. PREPARATION OF A SURFACE WITH COUNTERELECTRODES

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures in a square array pattern. A 10:1 mixture of SYLGARD silicone elastomer 184 and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD 184 is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a hydrophilic OH-terminated alkane thiol, $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$, in an ethanolic solution (1-10 mM), robotically brought into pin registered contact with an aligned patterned counterelectrode and square binding domain on a gold surface, and removed. The patterned gold surface consists of addressable ring counterelectrodes circumscribing the binding domains where the $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$ has been stamped. A gap or separation space exists between each gold counterelectrode and each square gold substrate for each monolayer binding domain. A capillary array containing binding reagent solutions is then robotically brought into pin registered contact with the aligned surface registering the capillaries with the $\text{SH}(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_6\text{OH}$ domains to place specific antibodies or nucleic acids on each domain. Each capillary in the capillary array contains antibodies or nucleic acids, specific for an analyte of interest, capable of covalently binding to the reactive OH groups on the hydrophilic domains.

6.17. ASSAY CONDUCTED ON A SINGLE SURFACE

WITH THE WORKING AND COUNTERELECTRODES ON DIFFERENT SURFACES

The support surface described above in example 6.16 is exposed to a sample solution to be analyzed. The support surface is then washed and exposed to a solution containing a plurality of ECL labelled monoclonal antibodies or ECL labelled nucleic acids of differing specificity and then washed with assay buffer containing tripropyl amine. A transparent addressable working electrode array is fabricated with each working electrode in the array corresponding to a discrete binding domain/counter-electrode region on the support as described above in Section 6.16. The two supports are wetted with the assay buffer and robotically brought into registered aligned conformal contact. The electrode arrays are connected to an electronic potential wave form generator, and potential is applied to the aligned working electrode/counter-electrode pairs creating a potential field between the two supports. A CCD then reads the light emitted through the transparent working electrode and the signal is sent to a microprocessor which converts the signal to the desired readout form.

The readout is compared to the readout obtained using controls in the form of known quantities of an analyte of interest to calculate the actual quantity of analyte.

6.18. FABRICATION OF A CC (DISPERSED) FIBRIL MAT BY VACUUM FILTRATION

An aqueous slurry of CC fibrils, with 1 mg fibrils/ mL solution was prepared by mixing 0.1 % w/w CC fibrils/ deionized water. The CC fibrils were dispersed (the larger, micron-scale aggregates were dispersed into small aggregates or individual fibers) in the slurry by immersing a 400 watt sonication horn in the slurry for between 10 minutes and 1 hour. The extent of dispersion was monitored by optical microscopy.

A nylon filter membrane (0.47 μ m pore size, 25 mm diameter) was placed in a 25 mm diameter glass fritted filter. The dispersed fibril slurry was filtered through the membrane/filter set-up by suction filtration (Fig. 23A).

Aliquots of the slurry (5 ml) were diluted with 20 ml deionized water, then filtered through the membrane/filter set-up. For an average mat of approximately 0.25-0.3 gram/cc, a mat of approximately 100 μm required 6 aliquots.

Suction filtration was continued until all of the water from the dispersion was removed from the mat (by visual inspection). The mat was peeled (by hand) directly from the filter membrane.

The mat was dried in an oven for approximately 10-15 minutes at 60°C. The mat was cut, punched or otherwise sectioned for use.

6.19. FABRICATION OF A FIBRIL MAT ON A METAL MESH SUPPORT BY EVAPORATION

An aqueous slurry of CC fibrils, with 1 mg fibrils/ mL solution was prepared by mixing 0.1 % w/w CC fibrils/deionized water. The CC fibrils were dispersed (the larger, micron-scale aggregates were dispersed into small aggregates or individual fibers) in the slurry by immersing a 400 watt sonication horn in the slurry for between 10 minutes and 1 hour. The extent of dispersion was monitored by optical microscopy.

A 1 cm² section of stainless steel mesh (400 count) was placed on a 25 mm diameter paper filter. A 5 ml aliquot of the slurry was pipetted onto the surface of the screen/filter paper ensemble. The water in the slurry was allowed to evaporate, either at room temperature and pressure, or in a heated oven.

Once the fibril mat was dry, additional aliquots were added. The fibril and screen were peeled as a single unit from the filter paper.

The mat was cut, punched or otherwise sectioned for use.

6.20. IMMOBILIZATION OF AVIDIN ON FIBRILS BEARING NHS-ESTER FUNCTIONAL GROUPS

Fibrils derivatized with COOH (provided by Hyperion Catalysts Inc.) were suspended in anhydrous dioxane at ~10 mg/ml with constant stirring. A 20-fold molar excess of N-hydroxysuccinimide was added and allowed to dissolve. Next, a

20-fold molar excess of ethyl-diamino-propyl-carbodiimide (EDAC) was added, and the mixture was stirred for 2 hours at room temperature.

After stirring, the supernatant was aspirated and the solids were washed three times with anhydrous dioxane, one time with anhydrous methanol, and filtered on a 0.45 μ m polysulfone membrane. The filtrate was washed with additional methanol and the placed in a glass vial under vacuum until no further reduction in weight was observed.

10.4 mg of NHS-ester fibrils were washed with PBS-1 (~70 mM phosphate, 150 mM NaCl) (ORIGEN reagent 402-130-01, pH 7.8, IGEN, Inc.). The washed fibrils were suspended in 2.3 ml solution of avidin (8.3 mg avidin/ml PBS-1).

The suspension was allowed to sit at room temperature for 1.5 hours, with constant rotation of the flask to provide agitation.

After, 1.5 hours, the suspension was stored for 16 hours at 4°C, then brought to room temperature and washed with PBS-1 and stored at 4°C as a suspension in PBS-1.

6.21. IMMOBILIZATION OF MONOCLONAL ANTIBODY (ANTI-AFP) ON CARBON FIBRILS

Carbon fibrils functionalized with NHS esters were prepared as described in Example 6.20.

14 mg of fibril-NHS ester was mixed with 500 ml PBS-1 buffer. The mixture was sonicated for 20 min, until it became a viscous slurry. An additional 500 ml of PBS-1 buffer was added.

A total of 1.6 mg anti-AFP (alpha-fetal protein) antibody in 80 ml PBS-1 was added to the above slurry. The reaction was allowed to sit at room temperature for 2.5 hours.

6 ml PBS-1 buffer was added and the reaction mixture was centrifuged at 4 °C for 5 minutes. The supernatant was removed by pipette. This procedure was repeated 9 times.

After the final wash, the supernatant was removed, and the fibril-antiAFP product was stored at 4°C.

6.22. CYCLIC VOLTAMMOGRAMS OF FIBRIL
MATS: COMPARISON OF FIBRIL MAT
WITH GOLD FOIL ELECTRODE

Cyclic voltammograms of 6 mM $\text{Fe}^{3+/2+}(\text{CN})_6$ in 0.5 M K_2SO_4 were measured. In Fig. 30A, the CV for a plain fibril mat of CC(dispersed) was measured at 0.10 mA/cm at 10, 25 and 50 mV/sec. The mat was fabricated as described in Example 6.18. In Fig. 30B, the CV was measured for a gold foil electrode at 0.05 mA/cm at 10, 25 and 50 mV/sec. All potentials are in Volts vs. Ag/AgCl.

6.23. ELECTROCHEMICAL PROPERTIES OF FIBRIL
MAT ELECTRODES: COMPARISON OF ANODIC
PEAK CURRENT WITH THICKNESS OF THE MAT

Cyclic voltammograms of 6 mM $\text{Fe}^{3+/2+}(\text{CN})_6$ in 0.5 M K_2SO_4 were measured for fibril mats of the same geometric area (0.20 cm^2), but different thicknesses. The anodic peak current (Fig. 31) increased with increasing thickness of mat for thicknesses that ranged from 24 μm to 425 μm . For each thickness, the anodic peak current also increased with increasing scan rates (for rates that ranged from 10 mV/sec to 150 mV/sec). The rate of increase of the anodic peak current, as a function of thickness, also increased with increasing thickness. Fibril mats that were 24 μm thick behaved comparably to gold foil electrodes.

6.24. NON-SPECIFIC BINDING OF PROTEINS TO FIBRILS

Non-specific binding of proteins to carbon fibrils (cc) was measured as follows: i) a solution of $\text{Ru}(\text{bipy})_3^{2+}$ ("TAG1") labeled proteins was exposed to a known quantity of carbon fibrils until equilibrium was reached; ii) the labeled-protein/fibril solution was centrifuged, and the supernatant was collected, and iii) the amount of labeled-protein remaining in the supernatant was assayed using electrochemiluminescence (ECL).

To generate the curve shown in Fig. 32, anti-CEA antibody attached to derivatized TAG1 (antibody to carcinoembryonic antigen attached to a derivatized TAG1 ECL label) at 3 $\mu\text{g/mL}$, was added to serial dilutions of CC(plain) fibrils in 0.1 M potassium phosphate buffer at pH 7. Fibrils were removed by centrifugation after vortexing for 20 minutes.

ECL assays that measured the amount of protein (unbound) remaining in the supernatant were run in an ORIGEN 1.5 (IGEN, Inc.) analyzer on aliquots of the reaction mixture supernatant diluted 5 times with ORIGEN assay buffer. A decrease in the ECL signal (relative to the ECL signal for an object of the reaction mixture that had not been exposed to fibrils) resulted from increased binding of protein labelled with a derivatized TAG1 when a higher concentration of carbon fibrils were present.

6.25. REDUCTION OF NON-SPECIFIC BINDING OF PROTEINS TO FIBRILS WITH DETERGENTS/SURFACTANTS

Using the method described in Example 6.2.4, the effect of surfactant on protein binding to fibrils was analyzed. Triton X-100 was added to the anti-CEA attached to derivatized TAG1/fibril mixture, the solution was incubated for 20 minutes, the tubes were centrifuged, and aliquots of the supernatant, diluted 5 times with ORIGEN assay buffer, were analyzed by ECL. The results are shown in the Table below and in Fig. 33.

Tube Number	[T-X100], ppm	Peak Intensity	Prot-TAG1 $\mu\text{g/ml}$	[GF], ppm
19	1674	1611	2.65	52
18	837	1634	2.65	52
17	418	1697	2.65	52
16	209	1583	2.65	52
15	105	1772	2.65	52
14	52	1463	2.65	52
13	26	627	2.65	52
12	13	23	2.65	52

A curve that results from plotting the ECL intensity of a protein labelled with a derivatized TAG1 in solution vs. Triton X-100 concentration is shown in Fig. 33. A higher ECL signal corresponds to more derivatized-TAG1-labeled protein in the supernatant, which corresponds to less derivatized-TAG1-labeled

protein bound to the fibrils. Concentrations of Triton x-100 that ranged from 10 ppm to 100 ppm reduced the extent of binding; increasing the concentration from 100 to 2000 ppm did not further reduce the extent of binding.

6.26. ECL OF FREE TAG IN SOLUTION WITH FIBRIL MAT ELECTRODE

A fibril mat prepared as in Example 6.18 was installed in the mounting area 3403 of the working electrode holder 3401 of the "Fibril Cell" fixture shown in Fig. 34. The holder 3401 was slipped into the bottom of the electrochemical cell compartment 3400. The 3 M Ag/AgCl reference electrode (Cyprus # EE008) was installed into the electrochemical cell compartment through the reference cell hole 3402. The cell was filled with Assay Buffer (IGEN # 402-005-01 lot# 5298) and attached to the PMT holder 3404. Using a EG&G PARC model 175 universal programmer and an EG&G model 175 Potentiostat/Galvanostat the potential was swept from 0 V to +3 V vs. Ag/AgCl at 100 mV/s. The ECL was measured by a Hamamatsu R5600U-01 which was powered at 900V by a Pacific Instruments model 126 Photometer. The analog data was digitized at 10 Hz by a CIO-DAS-1601 A/D board driven by HEM Snap-Master. The Fibril Cell was drained, flushed with 1000 pM TAG1 (IGEN # 402-004-C lot# 4297), and filled with 1000 pM TAG1. The potential was swept as with Assay Buffer. Shown in Fig. 35 are the ECL traces (measured at 24.0 ± 0.2 C) for Assay Buffer 3501 and 1000 pM TAG1 3502. The dark corrected ECL peak area was 22.10 nAs for Assay Buffer and 46.40 nAs for 1000 pM TAG1.

6.27. ECL OF ADSORBED LABELED ANTIBODY WITH FIBRIL MAT ELECTRODE

Fibril mats were made to a thickness of 0.0035 inches from plain cc-dispersed fibrils in the manner described in Example 6.18. The dried mats were then punched into 3mm disks and mounted onto supports. The supports used in this experiment were fabricated from 0.030 inches polyester sheet patterned by screen printed conductive gold ink. This conductive gold ink formed the counter electrode, reference electrode, and provided leads for the working and other electrodes. Two fibril mat disks

were mounted to each patterned support using two sided carbon containing conductive tape (Adhesives Research). After mounting, the disks were spotted with 0.5 μ l of 10 μ g/ml anti-TSH antibody attached to derivatized TAG1 in deionized water (Ru-TSH mono 1:2 26JUN95, IGEN, Inc.) or 0.5 μ l of 10 μ g/ml anti-TSH unTAG1'ed capture antibody in deionized water (TSH poly 25JUN95, IGEN, Inc.) and allowed to dry. After drying, the mats were flooded with IGEN assay buffer. Flooded mats on supports were loaded into an IGEN Origen 1.5 based instrument and ECL was read using a scan rate of 500mV/s from 0 to 4500mV. Fig. 43 compares the peak ECL signals from TAG1-antibody containing mats 4301 and unTAG1'ed capture antibody containing mats 4302.

6.28. ECL USING FIBRIL MAT
ELECTRODE FOR SANDWICH ASSAY

Anti-AFP capture antibody was immobilized on fibrils as described above. Anti-AFP fibrils were washed into deionized water (dI) and resuspended at a density of 1mg/ml. A four layer fibril mat was produced using vacuum filtration as described in Example 6.18. Two milligrams of anti-AFP fibrils were added to 3mg of plain CC dispersed fibrils and the mixture diluted to a total volume of 20 ml in dI. The diluted mixture was filtered onto a 0.45 μ m nylon filter. This initial mat layer was then followed by two core layers, each consisting of 5mg of plain CC dispersed fibrils. The mat core was then topped with a mixed fibril layer identical to the initial layer. This resulted in a fibril mat that was ~40% anti-AFP fibrils on the top and bottom surface and ~100% plain fibrils in the core. This mixed mat was air dried under vacuum and punched into 3mm disks. These disks were then mounted onto supports as described in Example 6.27. Dry, supported, anti-AFP mats were flooded with AFP calibrators A, C, and F (IGEN, Inc.) and allowed to incubate for 15 minutes at room temperature on the bench top. After incubation, supported electrodes were washed with a dI stream for 10 seconds and then blotted dry with a lint free wipe. Fibril mats were then flooded with anti-AFP attached to antibody labelled with derivatized TAG1 (IGEN, Inc.) and allowed to incubate for 15

minutes at room temperature on the bench top. After incubation the supported electrodes were washed with dI and dried with a wipe. Fibril mats were then flooded with IGEN assay buffer and read as described in Example 6.27.

6.29. ECL DETECTION OF TAG1-LABELED AVIDIN ON A POLYACRYLAMIDE SURFACE

A cross-linked polyacrylamide gel containing covalently bound biotin was prepared by copolymerization of acrylamide, bis-acrylamide, and *N*-Acryloyl-*N'*-biotinyl-3,6-dioxaoctane-1,9-diamine (biotin linked to an acrylamide moiety through a tri(ethylene glycol) linker) using well known conditions (initiation with ammonium persulfate and TEMED). In this experiment, the concentrations of the three monomeric species were 2.6 M, 0.065 M, and 0.023 M respectively (these concentrations of acrylamide and bis-acrylamide are reported to result in gels with pore sizes smaller than most proteins). Polymerization of the solution containing the monomers between two glass plates held apart to a distance of approximately 0.7 mm led to the formation of a slab gel with the same thickness. After the polymerization reaction was complete, any unincorporated biotin was washed out by soaking the gel in four changes of PBS. Avidin labeled with a derivatized TAG1 (where Avidin refers to NeutrAvidin, a modified avidin designed to exhibit reduced NSB, was used in this experiment) was bound to the surface of the gel by soaking the gel in a solution containing the protein at a concentration of 50 µg/mL in PBS for 20 min. Excess TAG1-labeled avidin was then washed away by soaking the gel in four changes of ECL assay buffer (200 mM sodium phosphate, 100 mM tripropylamine, 0.02% (w/v) Tween-20, pH 7.2). As shown in Fig. 39, the gel (3900) was then placed in contact with gold working (3901) and counter (3902) electrodes patterned on a glass support (3903). Ramping the potential across the two electrodes from 0.0 to 3.0 V and back to 0.0 V at a rate of 500 mV/s led to an ECL light signal as measured from a PMT (3904) placed above the gel (Fig. 40). A gel prepared without inclusion of the biotin containing acrylamide derivative

gave no ECL signal (Fig. 41). This signal obtained for the biotin-containing polymer was indicative of close to a full monolayer of protein is present on the surface of the gel.

6.30. ECL SANDWICH IMMUNOASSAY ON A POLYACRYLAMIDE SURFACE

A cross-linked polyacrylamide gel containing covalently bound biotin is prepared as described in Example 6.29. Streptavidin is adsorbed on the surface of the gel to form a binding domain capable of capturing biotin labeled species. The surface is treated with a solution containing tripropylamine, an unknown concentration of an analyte, a biotin-labeled antibody against the analyte, and a different ECL TAG1-labeled antibody against the analyte. The presence of the analyte causes the formation of a complex of the analyte and the two antibodies which is then captured on the streptavidin surface. ECL tag bound to secondary antibody present on the surface is measured as described in example 6.29

6.31. MULTIPLE ECL SANDWICH IMMUNOASSAYS ON POLYACRYLAMIDE SURFACES SUPPORTED ON AN ELECTRODE

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures to give a pattern of circular depressions arranged in an array. A 10:1 mixture of SYLGARD silicone elastomer 184 and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a solution containing the hydroxyl terminated thiol $\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_3-\text{OH}$ (1-10 mM) in ethanol, brought into contact with an aligned gold substrate and removed. The substrate is washed for several seconds with a solution containing the thiol $\text{HS}-(\text{CH}_2)_{10}-\text{CH}_3$ (1-10 mM in ethanol). The resulting surface is then rinsed with ethanol and dried under a stream of nitrogen. Treatment of the surface with a solution containing acryloyl chloride and triethylamine in dioxane leads to functionalization of the hydroxyl terminated domains with

acrylate groups. A capillary array containing mixtures of acrylamide, bis-acrylamide, N-acryloylsuccinimide, azo-bis-cyanovaleric acid, and antibodies presenting amino groups is then brought into contact with the aligned surface aligning the capillaries with the acrylate terminated domains to place prepolymer solutions containing specific antibodies at each domain. Each capillary in the capillary array contains antibodies specific for a different analyte of interest. Exposure of the patterned prepolymer droplets to UV light leads to the formation of cross-linked gels on the substrate each presenting a binding domain at the surface. The assay is carried out by treatment of the substrate with a mixture of analytes capable of binding at one or more of the binding domains presented on the gel surfaces in a buffered solution containing tripropylamine and ECL-TAG1 labeled secondary antibodies. The binding domains (4200, 4201, 4202) (on polyacrylamide drops (4203) on a gold electrode (4232) are then placed in close proximity to an ITO working electrode (4204) as shown in Figs. 42A-B. Light emitted from each of the binding domains is quantified using a CCD camera (4205) and compared to binding domains for internal standards included in the sample solution.

6.32. MULTIPLE ECL COMPETITIVE
IMMUNOASSAYS ON POLYACRYLAMIDE
SURFACES SUPPORTED ON AN ELECTRODE

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures to give a pattern of circular depression arranged in an array. A 10:1 mixture of SYLGARD silicone elastomer 184 and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a solution containing the hydroxyl terminated thiol $\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_3-\text{OH}$ (1-10 mM) in ethanol, brought into contact with an aligned gold substrate and removed. The substrate is washed for several seconds with a solution containing the thiol $\text{HS}-(\text{CH}_2)_{10}-\text{CH}_3$ (1-10 mM in ethanol). The

resulting surface is then rinsed with ethanol and dried under a stream of nitrogen. Treatment of the surface with a solution containing acryloyl chloride and triethylamine in dioxane leads to functionalization of the hydroxyl terminated domains with acrylate groups. A capillary array containing mixtures of acrylamide, bis-acrylamide, N-acryloylsuccinimide, azo-bis-cyanovaleric acid, and antibodies is then brought into contact with the aligned surface aligning the capillaries with the acrylate terminated domains to place prepolymer solutions containing specific antibodies at each domain. Capillaries in the capillary array contain antibodies specific for different analytes of interest. Exposure of the patterned prepolymer droplets to UV light leads to the formation of cross-linked gels on the substrate each presenting a binding domain at the surface. The assay is carried out by treatment of the substrate with a mixture of analytes capable of binding at one or more of the binding domains presented on the gel surfaces in a buffered solution containing tripropylamine and ECL-TAG1 labeled analogues of the analytes (i.e., setting up a competition of ECL-TAG1 labeled and unlabeled analytes for binding to the binding domains). The binding domains (4200, 4201 and 4202) (on polyacrylamide drops (4203) on a gold electrode (4232)) are then placed in close proximity to an ITO working electrode (4204) as shown in Fig. 42. Light emitted from each of the binding domains is quantified using a CCD camera (4205) and compared to binding domains for internal standards included in the sample solution.

6.33. MULTIPLE ECL ASSAYS FOR BINDING OF CELLS ON POLYACRYLAMIDE SURFACES SUPPORTED ON AN ELECTRODE

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures to give a pattern of circular depressions arranged in an array. A 10:1 mixture of SYLGARD silicone elastomer 184 and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by

exposure to a solution containing the hydroxyl terminated thiol $\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_3-\text{OH}$ (1-10 mM) in ethanol, brought into contact with an aligned gold substrate and removed. The substrate is washed for several seconds with a solution containing the thiol $\text{HS}-(\text{CH}_2)_{10}-\text{CH}_3$ (1-10 mM in ethanol). The resulting surface is then rinsed with ethanol and dried under a stream of nitrogen. Treatment of the surface with a solution containing acryloyl chloride and triethylamine in dioxane leads to functionalization of the hydroxyl terminated domains with acrylate groups. A capillary array containing mixtures of acrylamide, bis-acrylamide, N-acryloylsuccinimide, azo-bis-cyanovaleric acid, and antibodies directed against cell surfaces is then brought into contact with the aligned surface aligning the capillaries with the acrylate terminated domains to place prepolymer solutions at each domain. Exposure of the patterned prepolymer droplets to UV light leads to the formation of cross-linked gels on the substrate each presenting a binding domain at the surface. The assay is carried out by treatment of the binding domains first with a suspension of cells, then with a mixture of binding reagents capable of binding one or more of the cells bound to the gel surfaces in a buffered solution containing tripropylamine and ECL-TAG1 labeled secondary antibodies and/or other binding reagents specific for the analytes. The binding domains (4200, 4201, and 4202) (on polyacrylamide drops (4203) on a gold electrode (4232) are then placed in close proximity to an ITO working electrode (4204) as shown in Fig. 42. Light emitted from each of the binding domains is quantified using a CCD camera (4205) and compared to binding domains for internal standards included in the sample solution.

6.34. MULTIPLE ECL ASSAYS FOR BINDING OF ANALYTES TO CELLS ON POLYACRYLAMIDE SURFACES SUPPORTED ON AN ELECTRODE

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures to give a pattern of circular depressions arranged in an array. A 10:1 mixture of SYLGARD silicone elastomer 184 and the

corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a solution containing the hydroxyl terminated thiol $\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_3-\text{OH}$ (1-10 mM) in ethanol, brought into contact with an aligned gold substrate and removed. The substrate is washed for several seconds with a solution containing the thiol $\text{HS}-(\text{CH}_2)_{10}-\text{CH}_3$ (1-10 mM in ethanol). The resulting surface is then rinsed with ethanol and dried under a stream of nitrogen. Treatment of the surface with a solution containing acryloyl chloride and triethylamine in dioxane leads to functionalization of the hydroxyl terminated domains with acrylate groups. A capillary array containing mixtures of acrylamide, bis-acrylamide, N-acryloylsuccinimide, azo-bis-cyanovaleric acid, and cells is then brought into contact with the aligned surface aligning the capillaries with the acrylate terminated domains to place prepolymer solutions containing specific cell types at each domain. Capillaries in the capillary array contain cells with different surface structures that bind different analytes. Exposure of the patterned prepolymer droplets to UV light leads to the formation of cross-linked gels on the substrate each presenting a binding domain at the surface. The assay is carried out by treatment of the gels with a sample containing a mixture of analytes capable of binding at one or more of the binding domains presented on the gel surfaces in a buffered solution containing tripropylamine and ECL-Tag labeled antibodies and/or other binding reagents specific for the analytes. The binding domains (4200, 4201 and 4202) (on polyacrylamide drops (4203) on a gold electrode (4232) are then placed in close proximity to an ITO working electrode (4204) as shown in Fig. 42. Light emitted from each of the binding domains is quantified using a CCD camera (4205) and compared to binding domains for internal standards included in the sample solution.

6.35. MULTIPLE ECL COMPETITIVE HYBRIDIZATION
ASSAYS ON POLYACRYLAMIDE SURFACES
SUPPORTED ON AN ELECTRODE

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures to give a pattern of circular depression arranged in an array. A 10:1 mixture of SYLGARD silicone elastomer 184 and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a solution containing the hydroxyl terminated thiol $\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_3-\text{OH}$ (1-10 mM) in ethanol, brought into contact with an aligned gold substrate and removed. The substrate is washed for several seconds with a solution containing the thiol $\text{HS}-(\text{CH}_2)_{10}-\text{CH}_3$ (1-10 mM in ethanol). The resulting surface is then rinsed with ethanol and dried under a stream of nitrogen. Treatment of the surface with a solution containing acryloyl chloride and triethylamine in dioxane leads to functionalization of the hydroxyl terminated domains with acrylate groups. A capillary array containing mixtures of acrylamide, bis-acrylamide, N-acryloylsuccinimide, azo-bis-cyanovaleric acid, and nucleic acid probes functionalized with amino groups is then brought into contact with the aligned surface, aligning the capillaries with the acrylate terminated domains to place prepolymer solutions containing specific probes at each domain. Capillaries in the capillary array contain probes specific for a nucleic acid sequence of interest. Exposure of the patterned prepolymer droplets to UV light leads to the formation of cross-linked gels on the substrate, each presenting a binding domain at the surface. The assay is carried out by treatment of the substrate with a sample mixture that may contain sequences capable of binding at one or more of the binding domains presented on the gel surfaces in a buffered solution containing tripropylamine and ECL-Tag1 labeled sequences which can compete with the analytes of interest for binding to the surface. The binding domains (4200, 4201, and 4202) (on

polyacrylamide drops (4203) on a gold electrode (4232) are then placed in close proximity to an ITO working electrode (4204) as shown in Fig. 42. Light emitted from each of the binding domains is quantified using a CCD camera (4205) and compared to binding domains for internal standards included in the sample solution.

6.36. MULTIPLE ECL HYBRIDIZATION SANDWICH .
ASSAYS ON POLYACRYLAMIDE SURFACES
SUPPORTED ON AN ELECTRODE

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures to give a pattern of circular depression arranged in an array. A 10:1 mixture of SYLGARD silicone elastomer 184 and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a solution containing the hydroxyl-terminated thiol $\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_3-\text{OH}$ (1-10 mM) in ethanol, brought into contact with an aligned gold substrate and removed. The substrate is washed for several seconds with a solution containing the thiol $\text{HS}-(\text{CH}_2)_{10}-\text{CH}_3$ (1-10 mM in ethanol). The resulting surface is then rinsed with ethanol and dried under a stream of nitrogen. Treatment of the surface with a solution containing acryloyl chloride and triethylamine in dioxane leads to functionalization of the hydroxyl terminated domains with acrylate groups. A capillary array containing mixtures of acrylamide, bis-acrylamide, N-acryloylsuccinimide, azo-bis-cyanovaleric acid, and nucleic acid probes functionalized with amino groups is then brought into contact with the aligned surface, aligning the capillaries with the acrylate terminated domains to place prepolymer solutions containing specific probes at each domain. Capillaries in the capillary array contain probes specific for a nucleic acid sequence of interest. Exposure of the patterned prepolymer droplets to UV light leads to the formation of cross-linked gels on the substrate, each presenting a binding domain at the surface. The assay is carried out by treatment of the substrate with a sample mixture that may contain

sequences capable of binding at one or more of the binding domains presented on the gel surfaces in a buffered solution containing tripropylamine and ECL-TAG1 labeled sequences which can bind the analytes at sequences not complementary to the surface-bound probes. The binding domains (4200, 4201, and 4202) (on polyacrylamide drops (4203) on a gold electrode (4232) are then placed in close proximity to an ITO working electrode (4204) as shown in Fig. 42. Light emitted from each of the binding domains is quantified using a CCD camera (4205) and compared to binding domains for internal standards included in the sample solution.

6.37. MULTIPLE ASSAYS OF DIFFERENT
TYPES ON A POLYACRYLAMIDE
SURFACES SUPPORTED ON AN ELECTRODE

An exposed and developed photoresist master of 1-2 microns thickness is prepared according to well known procedures to give a pattern of circular depressions arranged in an array. A 10:1 mixture of SYLGARD silicone elastomer 184 and the corresponding SYLGARD 184 curing agent is poured over the master and cured. The polymerized SYLGARD is carefully removed from the silicon master. The resulting elastomeric stamp is "inked" by exposure to a solution containing the hydroxyl terminated thiol $\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_3-\text{OH}$ (1-10 mM) in ethanol, brought into contact with an aligned gold substrate and removed. The substrate is washed for several seconds with a solution containing the thiol $\text{HS}-(\text{CH}_2)_{10}-\text{CH}_3$ (1-10 mM in ethanol). The resulting surface is then rinsed with ethanol and dried under a stream of nitrogen. Treatment of the surface with a solution containing acryloyl chloride and triethylamine in dioxane leads to functionalization of the hydroxyl terminated domains with acrylate groups. A capillary array containing mixtures of acrylamide, bis-acrylamide, N-acryloylsuccinimide, azo-bis-cyanovaleric acid, and any of the binding reagents described in Examples 6.31-6.36 is then brought into contact with the aligned surface aligning the capillaries with the acrylate terminated domains to place prepolymer solutions containing specific probes

at each domain. Each capillary in the capillary array contains binding domains specific for analytes of interest. Exposure of the patterned prepolymer droplets to UV light leads to the formation of cross-linked gels on the substrate each presenting a binding domain at the surface. The assay is carried out by treatment of the substrate with a sample mixture that may contain analytes capable of binding at one or more of the binding domains presented on the gel surfaces in a buffered solution containing tripropylamine and either ECL-TAG1 labeled analogues of analytes which compete with analytes for binding to the binding domains and/or ECL-TAG1 labeled secondary binding reagents to the analytes of interest. The binding domains (4200, 4201, and 4202) (on polyacrylamide drops (4203) on a gold electrode (4232) are then placed in close proximity to an ITO working electrode (4204) as shown in Fig. 42. Light emitted from each of the binding domains is quantified using a CCD camera (4205) and compared to binding domains for internal standards included in the sample solution.

6.38. HIGHLY REVERSIBLE ECL

Polycrystalline gold electrodes (purchased from Bio-Analytical Services, 2 mm²) were cleaned by hand polishing sequentially with 0.5 μ m and 0.03 μ m alumina slurry, followed by chemical etching in 1:3 H₂O₂/H₂SO₄ and electrochemical cycling in dilute H₂SO₄ between -0.2 V and 1.7 V vs. Ag/AgCl. The clean electrodes were then immersed overnight in a dilute solution of octylthiol (C₈SH) dissolved in ethanol. Protein adsorption was carried out by covering C₈SH-modified electrodes with 20 μ l of TAG1-labeled bovine serum albumin (BSA) dissolved in phosphate buffer saline (PBS, 0.15 M NaCl/0.1 M NaPi, pH 7.2) and washing the surface extensively with the same buffer after ten minute incubation.

ECL was done in a three-electrode cell with a Ag/AgCl reference electrode, platinum wire counter electrode and an EG&G 283 potentiostat. The light intensity was measured with a Pacific Instruments photometer and a Hamamatsu photo-multiplier tube

placing at the bottom of the electrochemistry cell. The protein-adsorbed electrode was immersed in a solution of 0.1 M TPA and 0.2 M phosphate, pH 7.2. Highly reversible ECL response (substantially similar intensity on the forward and backward scans) was observed when the electrode potential was cycled between 0.0 V and 1.2 V, as shown in Fig. 44A, indicating the reversible nature of the ECL process and stability of the thiol and protein layers on the electrode.

Cyclic voltammetric experiments were conducted on the same instruments as for ECL, without the use of PMT and photometer. In the experiment, a C₆SH-covered electrode (no protein) was placed in a solution of 1 mM potassium ferricyanide (in PBS) and the electrode was scanned from + 0.5 V to 1.2 V and back, followed by another cycle between + 0.5 V and - 0.3 V. It is indicative that the monolayer is still intact and not desorbing at 1.2 V, since there was only capacitive current in the voltammogram between + 0.5 V and - 0.3 V and no faradaic current of ferricyanide (Fig. 44B).

6.39. QUASI-REVERSIBLE ECL

Electrode modification and protein adsorption were done in the same way as described above. In the ECL experiments, the potential was scanned between 0.0 V and 1.5 V, and the corresponding light intensity was recorded. As illustrated in Fig. 45A, there was some loss of ECL between the forward and backward scans of the same cycle, as well as between different cycles. Cyclic voltammograms of the thiol/Au in ferricyanide after oxidizing at 1.5 V showed a significant amount of faradaic current, indicative of at least partial desorption of the thiol monolayer at 1.5 V (Fig. 45B).

6.40. IRREVERSIBLE ECL

In these experiments, electrode modification and protein adsorption were conducted in the same way as in Example 6.38. To measure ECL, the electrode potential was scanned all the way up to 2.0 V and back to 0.0 V. Intense light was observed on the forward scan (more light than was observed under

reversible conditions in Example 6.38), but it dropped to the background on the reverse scan, as shown in Fig. 46A. Cyclic voltammograms of the modified electrode in ferricyanide after oxidizing at 2.0 V indicated most of the thiol monolayer was desorbed (Fig. 46B).

6.41. AN ECL SANDWICH IMMUNOASSAY USING
A PRIMARY ANTIBODY IMMOBILIZED ON
A PATTERNED GOLD ELECTRODE

In this example an antibody against prostate specific antigen (PSA) is immobilized on a patterned gold electrode for use in an immunoassay for PSA.

An exposed and developed photoresist master of one to two microns thickness is prepared according to well known procedures to give a layer of photoresist on a silicon support with a 1mm x 1mm square patch where photoresist is removed. A 10:1 mixture of SYLGARD silicone elastomer 184 and the corresponding curing agent is poured over the master and cured. The polymerized SYLGARD is carefully removed from the silicon master. The resulting elastomeric "stamp" is "inked" by exposing it to a solution containing the hydroxyl-terminated thiol HS-(CH₂)₁₁(OCH₂CH₂)₃-OH and the nitrilotriacetic acid (NTA) terminated-thiol HS-(CH₂)₁₁(OCH₂CH₂)₃OC(O)NH(CH₂)₄CH(CO₂H)N(CH₂CO₂H)₂ in ethanol. The "inked" stamp is brought into contact with a gold substrate and removed to form a 1mm x 1mm SAM. The substrate is washed for several seconds with a solution containing only the hydroxyl-terminated thiol in ethanol, to prevent non-specific binding of proteins to the regions outside the stamped feature. The resulting surface is then rinsed with ethanol and dried under a stream of nitrogen. Treatment of the surface with a solution of NiCl₂ followed by treatment with a solution containing a fusion protein presenting the binding sites of an anti-PSA mouse monoclonal and the peptide (His)₆, leads to immobilization of the fusion protein on the surface in a controlled manner. This process yields a reproducible and predetermined amount of immobilized protein on the surface. The orientation of the

protein on the surface is controlled by the location of the (His)₆ sequence in the primary structure of the fusion protein. The absolute amount of immobilized protein is controlled by the ratio of NEA terminated-thiol to hydroxy-terminated thiol in the stamped SAM and by the surface area of the stamped feature. A calibration curve for PSA is determined by preparing solutions containing known concentrations of PSA in serum (at concentrations ranging from 1 fM to 1 uM. A number of surfaces prepared as described above are treated with the PSA calibration standards and then with a solution containing a secondary antibody against PSA (labeled with a derivative of TAG1) at an optimized concentration. A calibration curve is determined by immersing the surfaces in a solution containing 0.1 M TPA and 0.2 M phosphate (pH 7.2), and measuring the peak intensity of light emitted when the electrical potential at the gold surface is cycled between 0.0 and 2.0 V at a scan rate of 0.5V/sec. The determination of unknown concentrations of PSA in serum in a sample is conducted by the same procedure except that the concentration of PSA is calculated from the peak ECL signal by reference to the calibration curve.

7. INCORPORATION OF REFERENCES

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the forgoing description and accompanying figures. Such modifications are intended to fall within the scope of the claims. Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

We claim:

1. A method for conducting a plurality of electrochemiluminescence assays comprising the steps of:

(a) contacting a plurality of discrete binding domains with a sample containing one or a plurality of analytes of interest under assay conditions;

(b) causing one or more of said plurality of domains to electrochemiluminescence; and

(c) detecting electrochemiluminescence at a plurality of said binding domains.

2. The method of claim 1, wherein said plurality of binding domains includes binding domains having different binding specificities to provide for simultaneous binding of a plurality of different analytes of interest present in a sample.

3. An article comprising a plurality of discrete binding domains on a support, said binding domains having a relative spatial organization with respect to one another and having different binding specificities for binding a plurality of different analytes of interest in an electrochemiluminescence assay.

4. A cassette for use in the detection of an analyte in a sample by electrochemiluminescence comprising:

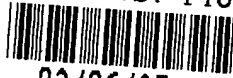
(a) a plurality of discrete binding domains on a support; and

(b) one or more pairs of electrodes and counterelectrodes.

5. A cassette as recited in claim 4 wherein said plurality of discrete binding domains forms at least one surface capable of binding a component of a binding electrochemiluminescence assay.

6. A cassette as recited in claim 4 wherein said plurality of binding domains includes binding domains having different binding specificities to provide for simultaneous binding of a plurality of different analytes of interest present in a sample.

7. A cassette as recited in claim 4 in which said discrete binding domains further comprise an internal control.



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ABSTRACT

Materials and methods are provided for producing patterned multi-array, multi-specific surfaces which are electronically excited for use in electrochemiluminescence based tests. Materials and methods are provided for the chemical and/or physical control of conducting domains and reagent deposition for use in flat panel displays and multiply specific testing procedures.

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For: MULTI-ARRAY, MULTI-SPECIFIC ELECTROCHEMILUMINESCENCE TESTING

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am

- ☒ the owner of the small business concern identified below:
- ☐ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN

MESO SCALE TECHNOLOGIES, LLC

ADDRESS OF CONCERN

1013 CENTRE ROAD, WILMINGTON, DELAWARE 19805

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees to the United States Patent and Trademark Office, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled MULTI-ARRAY, MULTI-SPECIFIC ELECTROCHEMILUMINESCENCE TESTING by inventor(s) JACOB WOHLSTADTER ET AL. described in

- ☒ the specification filed herewith.
- ☐ application serial no. _____, filed MARCH 6, 1996.
- ☐ patent no. __, issued __.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities (37 CFR 1.27).

RECEIVED "T" 030697

Applicant or Patentee: MESO SCALE TECHNOLOGIES, CURTIS, MORRIS & SAFFORD, P.C.
LLC

Serial or Patent No.:

File No.: 370068-6157

Filed or Issued:

MARCH 6, 1996

Page 2 of 2

For: MULTI-ARRAY, MULTI-SPECIFIC ELECTROCHEMILUMINESCENCE TESTING

FULL NAME

ADDRESS

☐ INDIVIDUAL

☐ SMALL BUSINESS CONCERN

☐ NONPROFIT ORGANIZATION

FULL NAME

ADDRESS

☐ INDIVIDUAL

☐ SMALL BUSINESS CONCERN

☐ NONPROFIT ORGANIZATION

FULL NAME

ADDRESS

☐ INDIVIDUAL

☐ SMALL BUSINESS CONCERN

☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING

JACOB WOHLSTADTER

TITLE OF PERSON
(if other than owner)

PRESIDENT

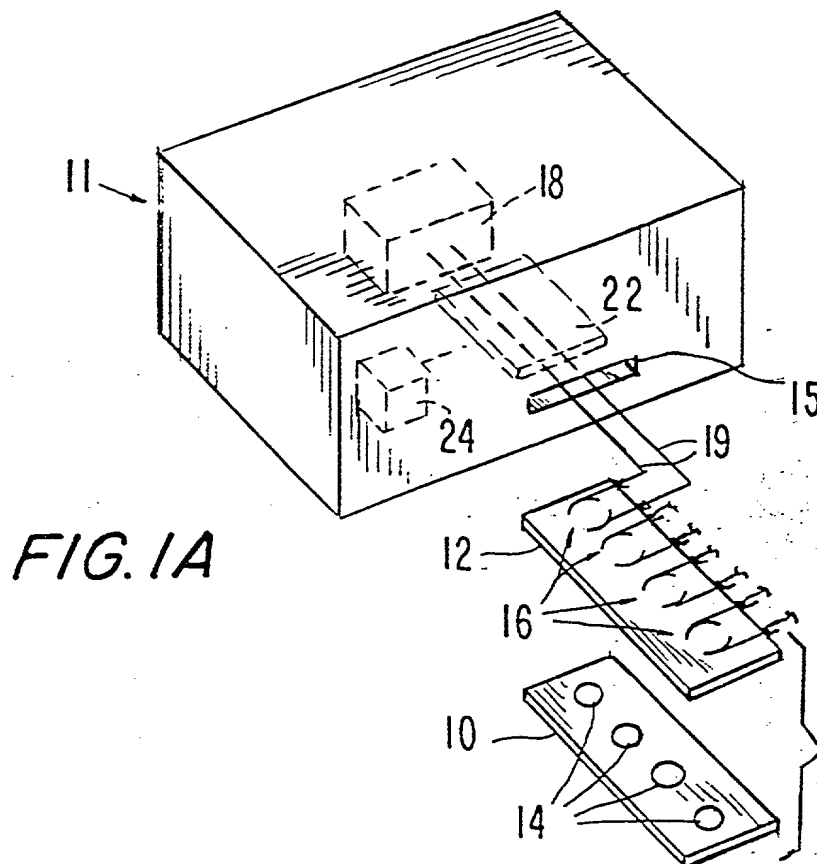
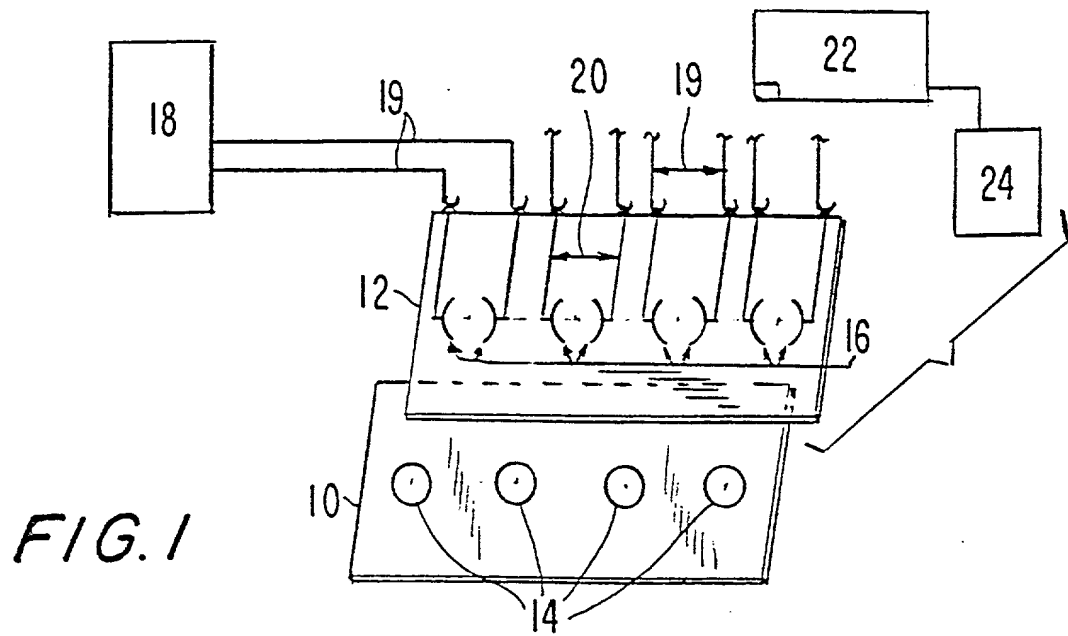
ADDRESS OF PERSON SIGNING

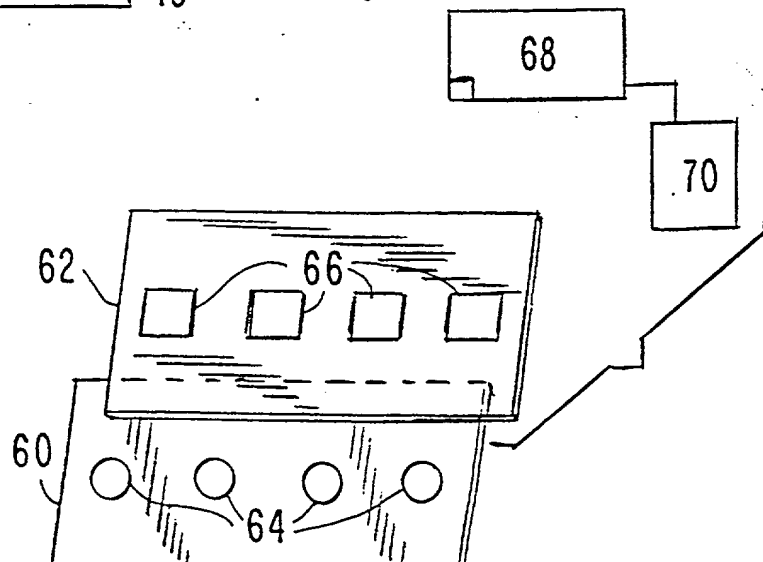
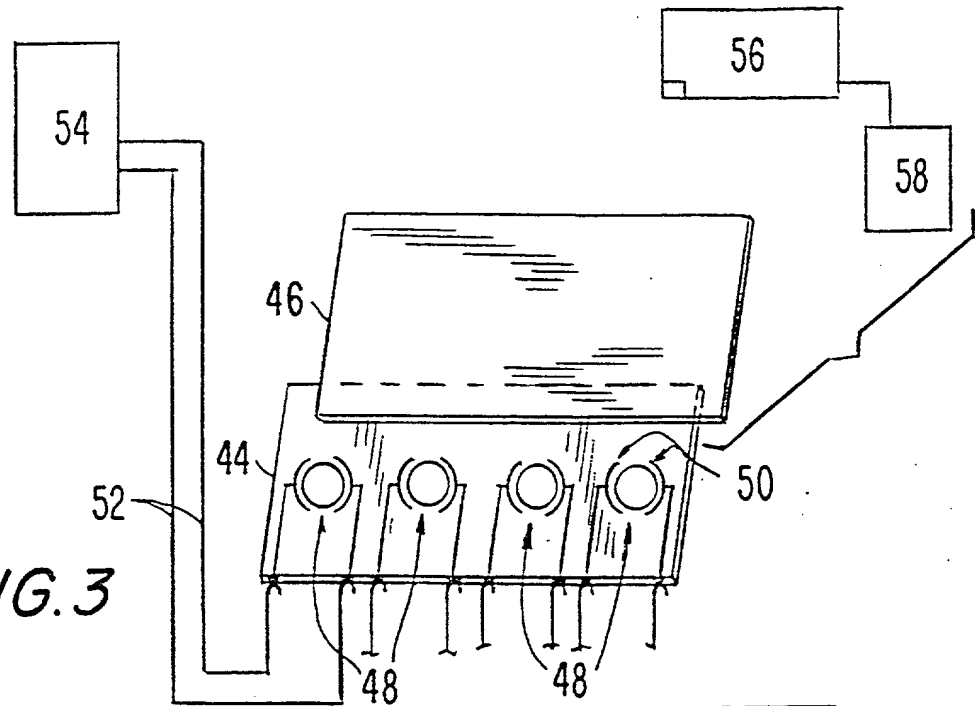
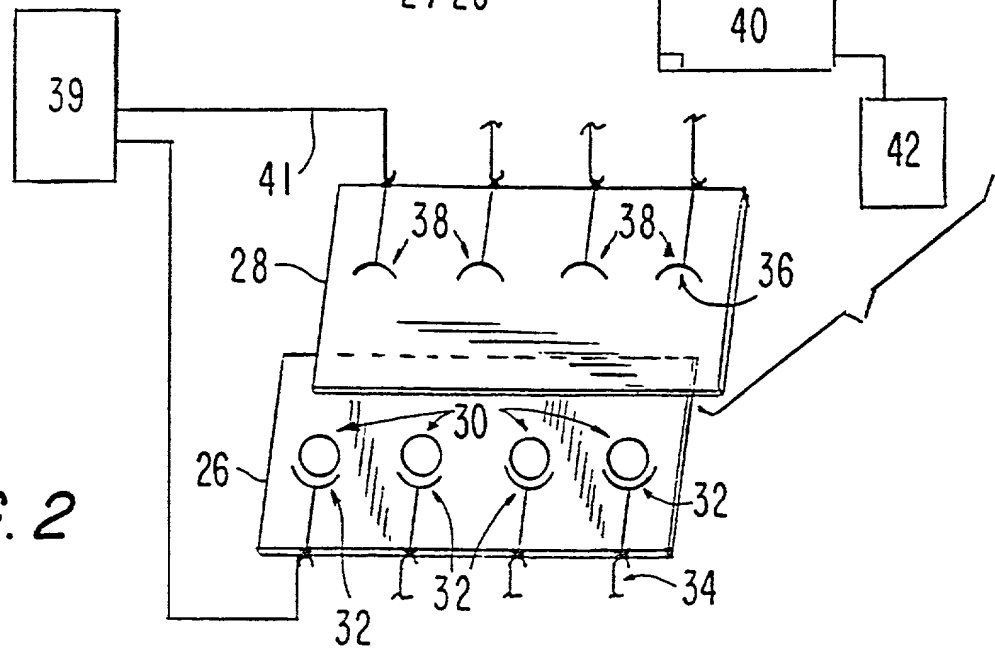
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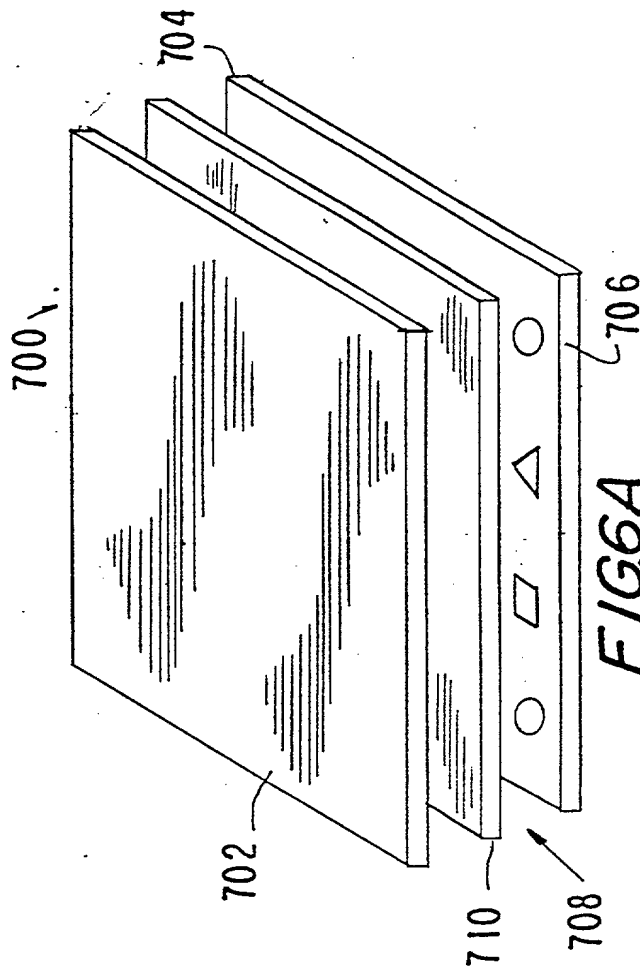
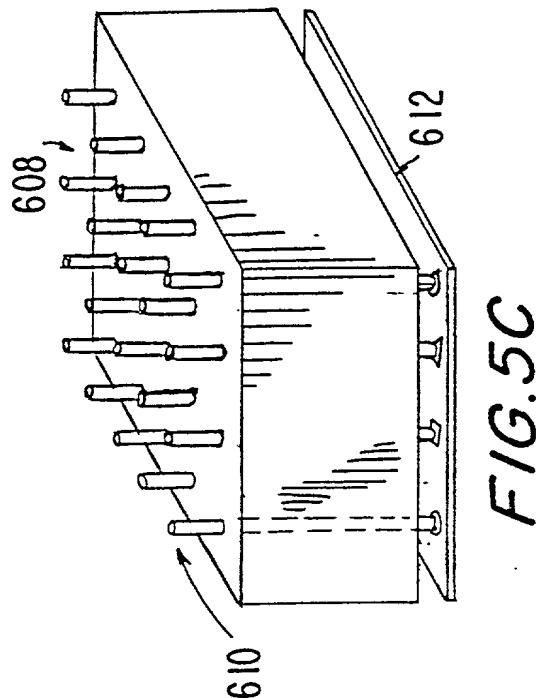
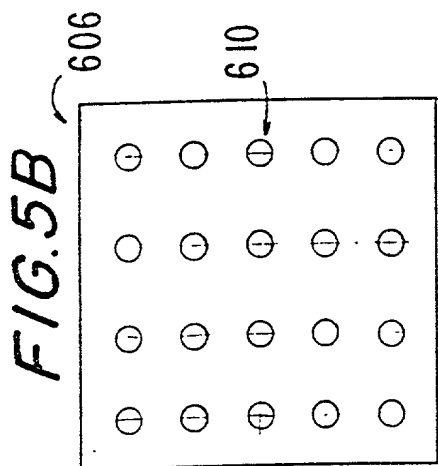
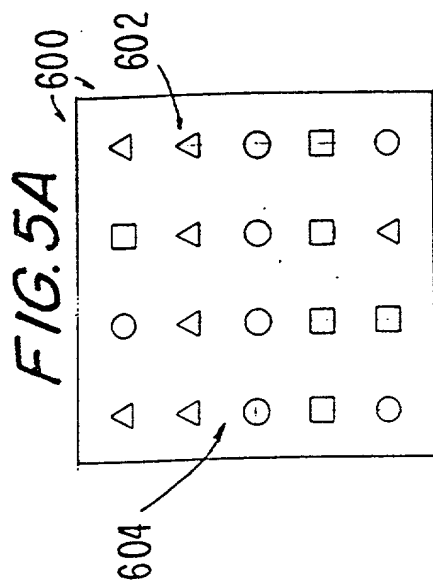
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DATE

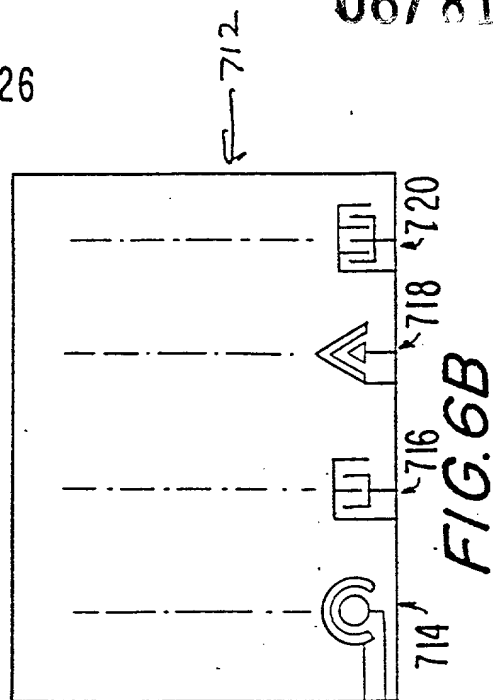
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FIG. 7

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800

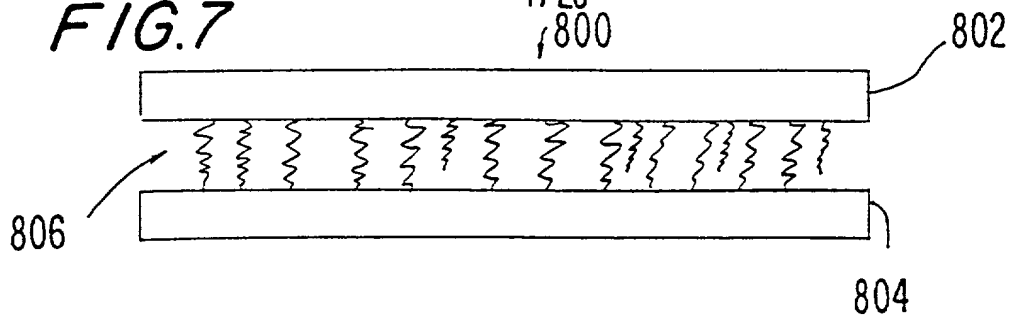


FIG. 8

900

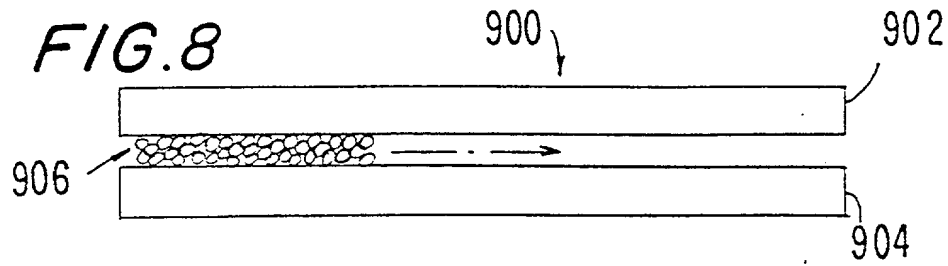


FIG. 9

1000

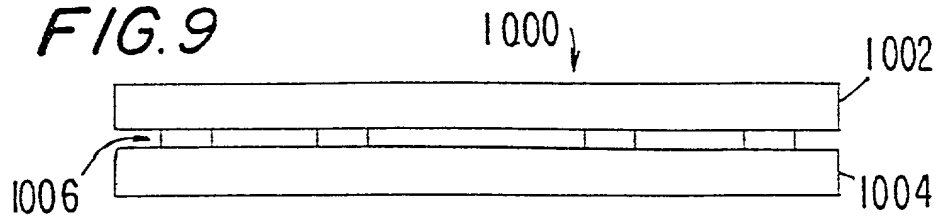
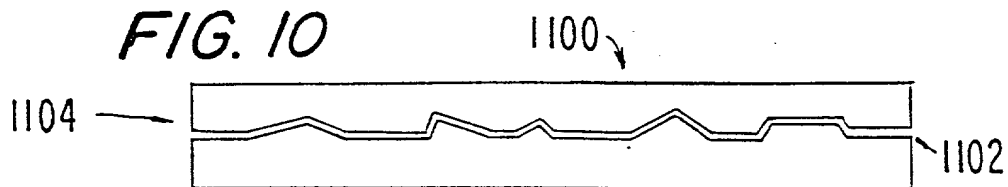
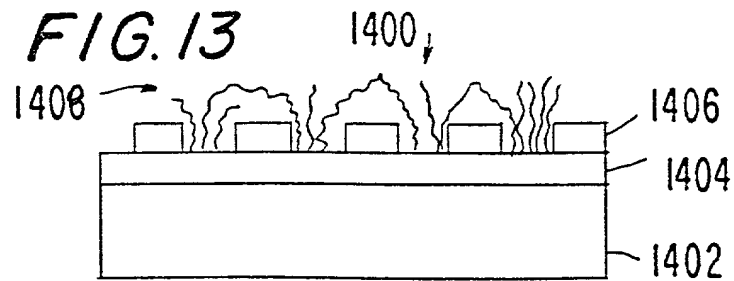
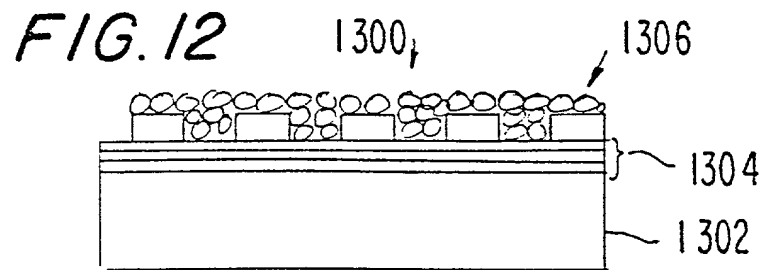
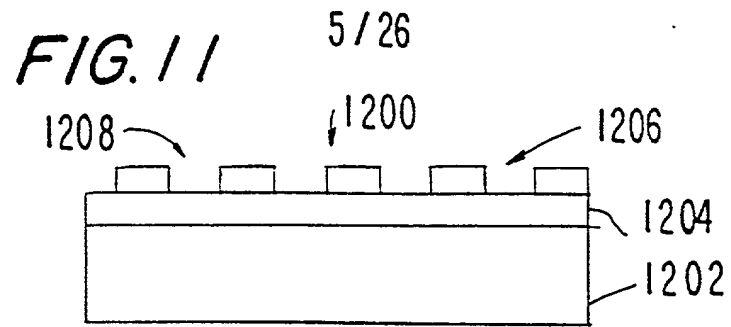


FIG. 10

1100





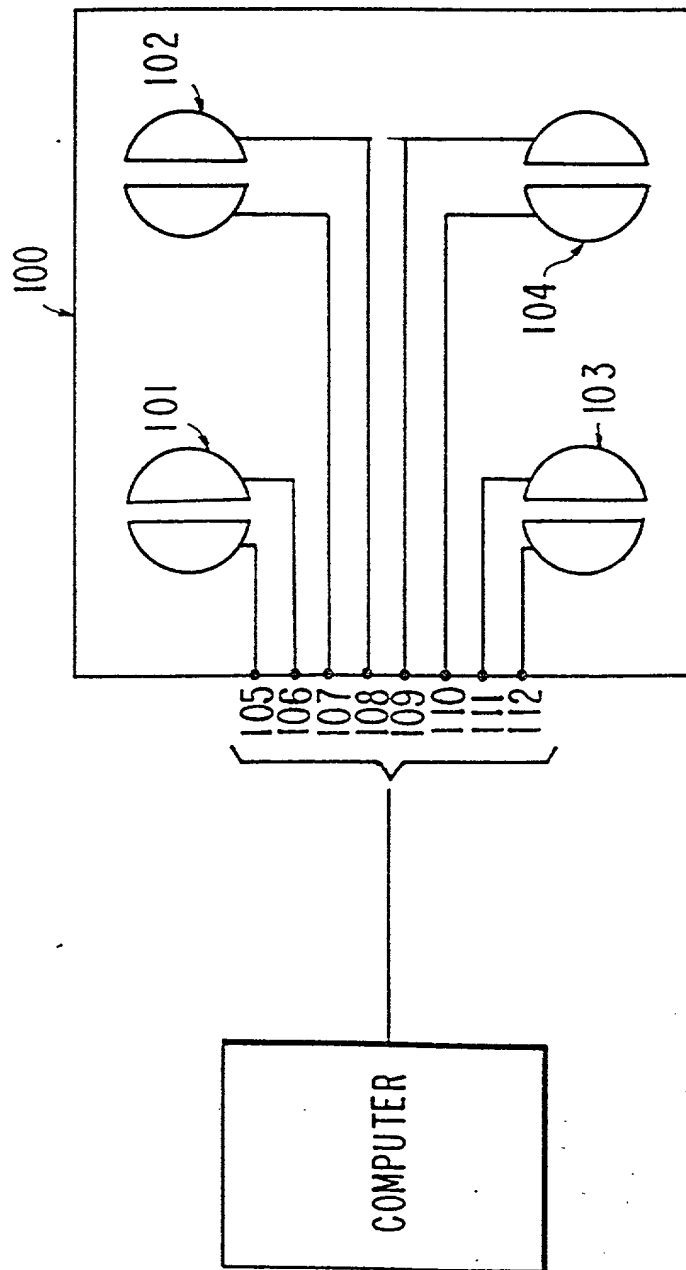


FIG. 14

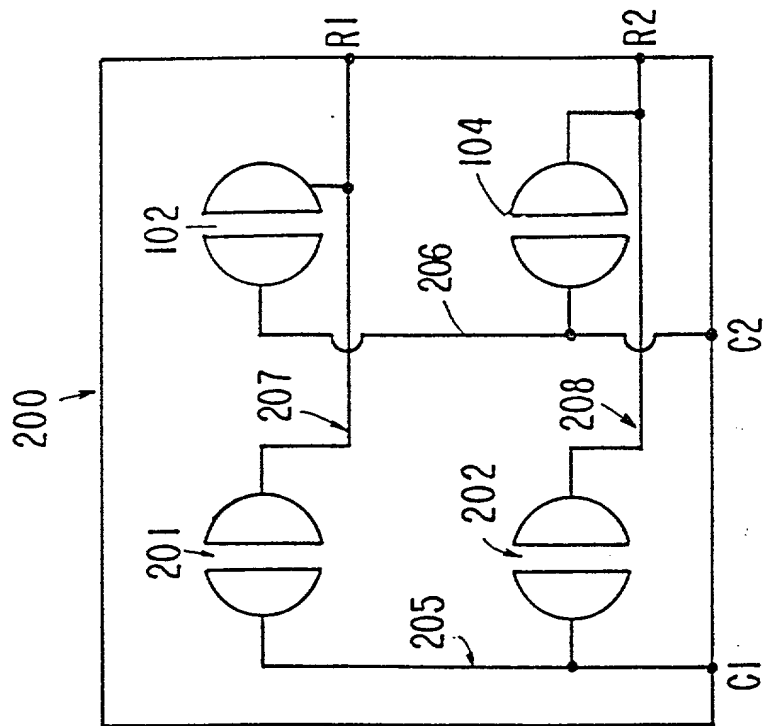


FIG. 15

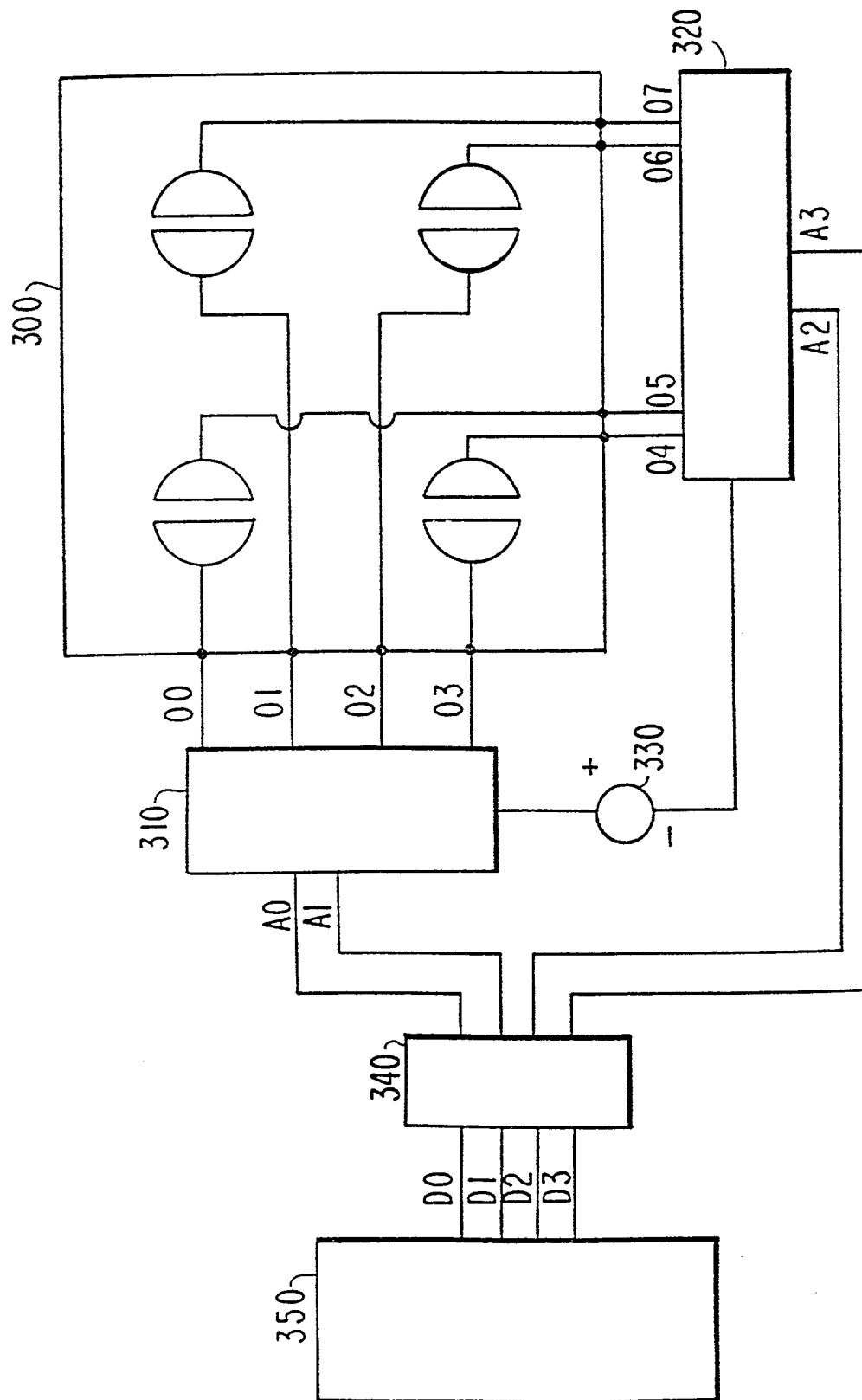


FIG. 16

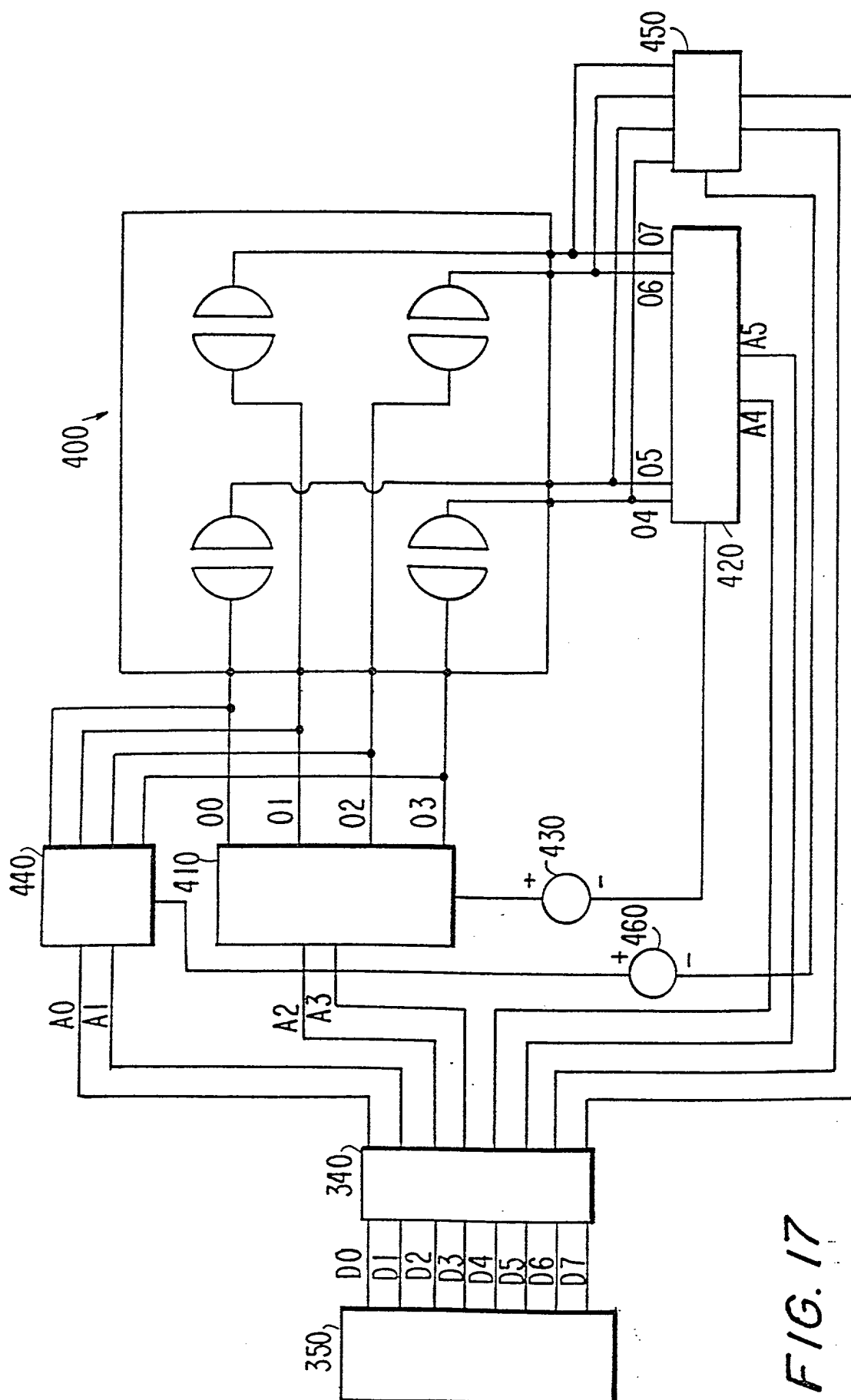


FIG. 17

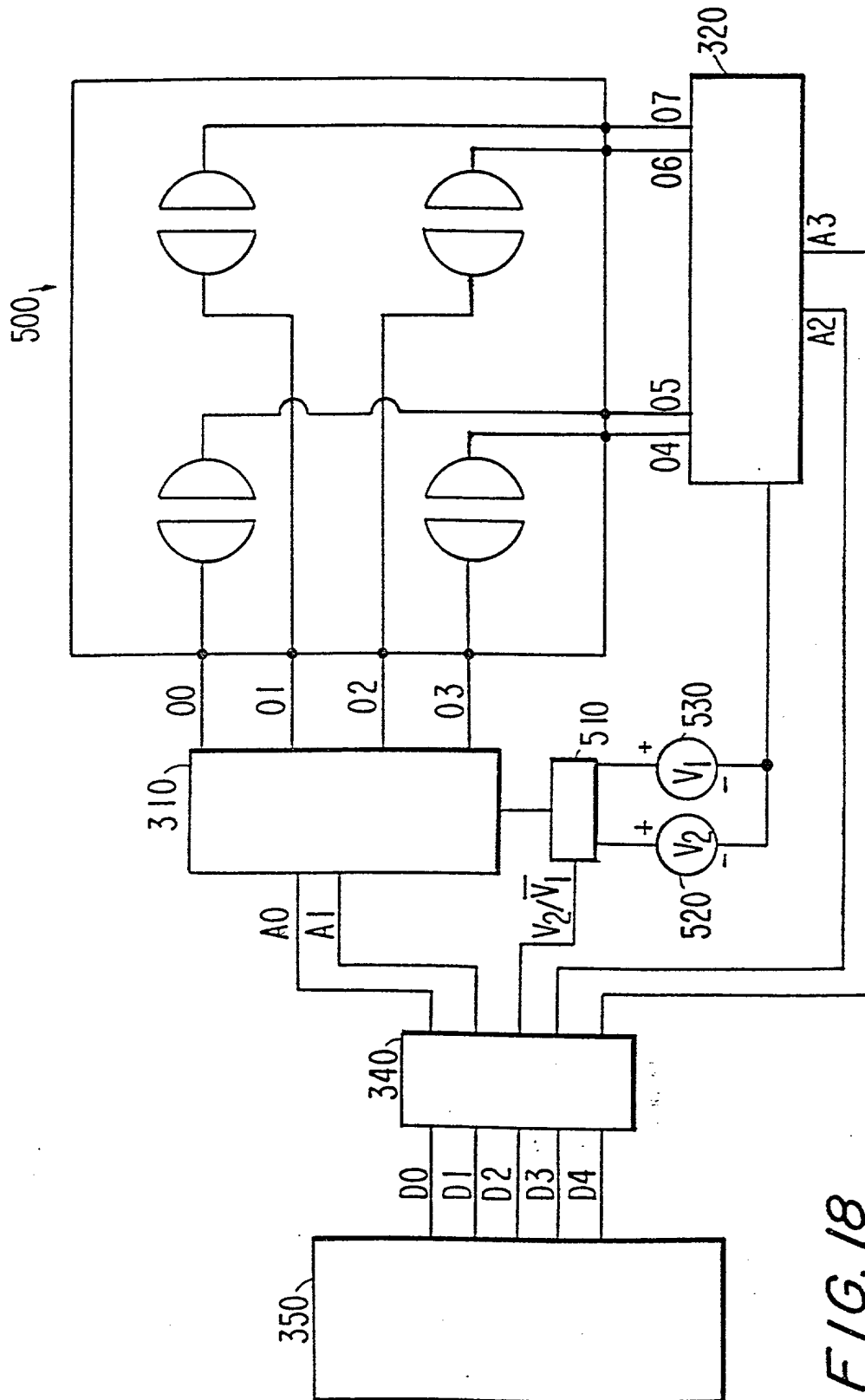


FIG. 18

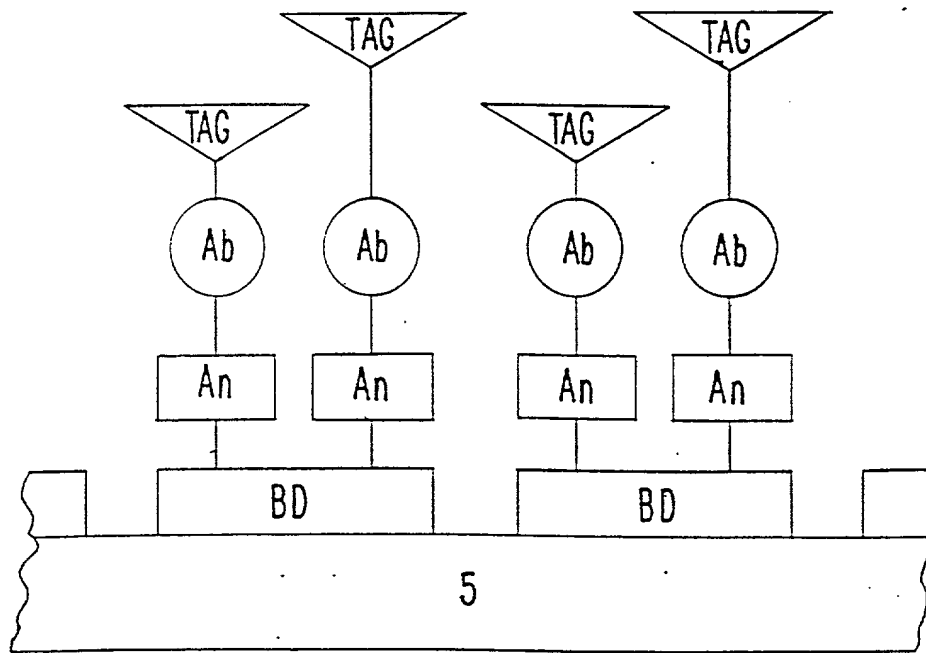
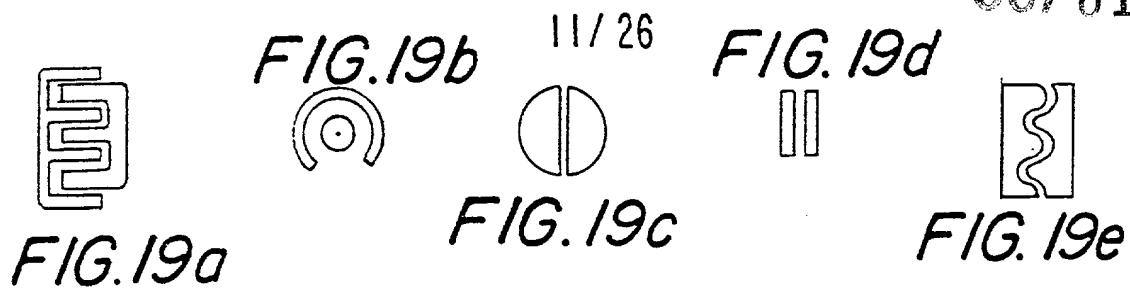


FIG. 20

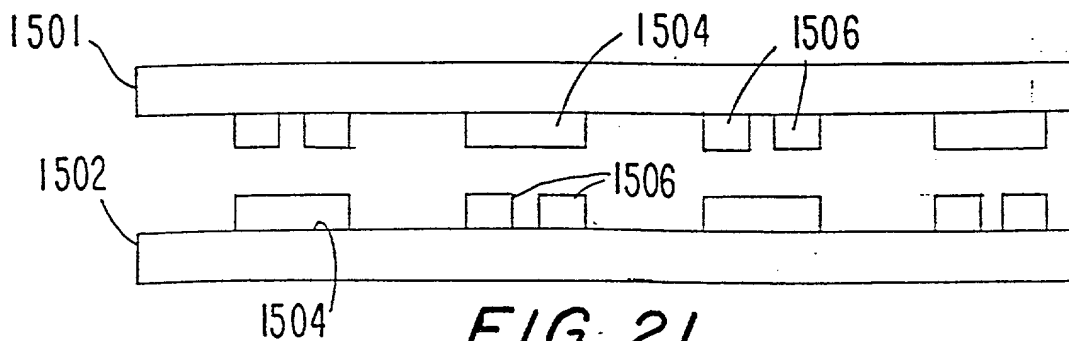


FIG. 21

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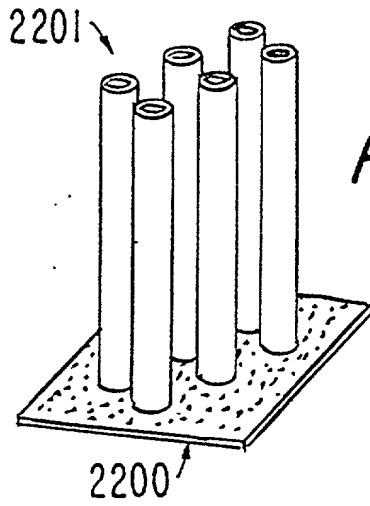


FIG. 22A

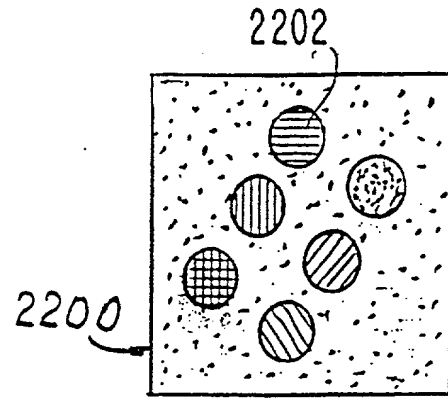


FIG. 22B

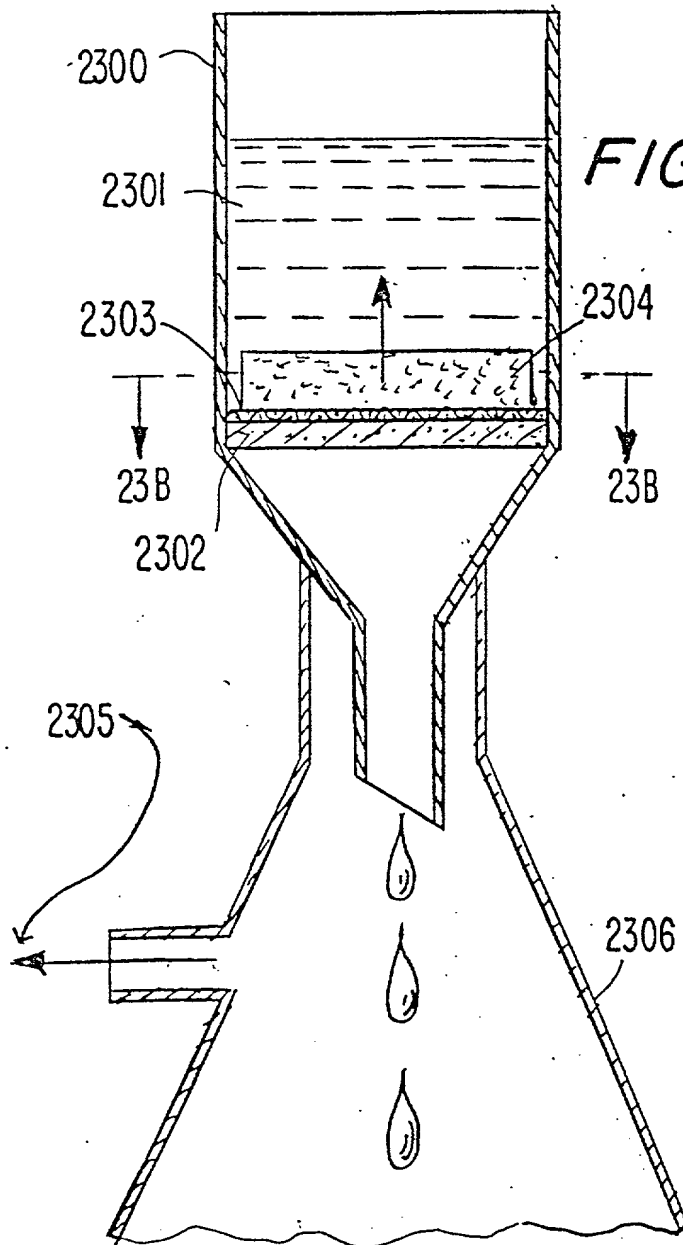


FIG. 23A

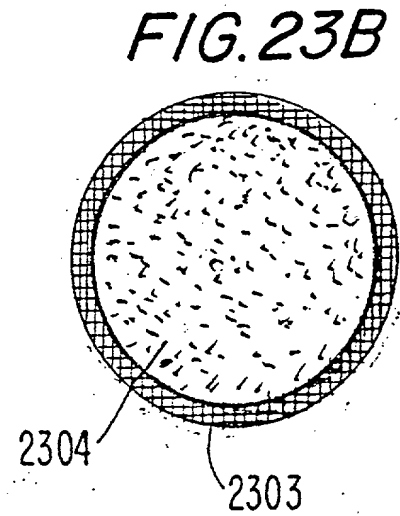


FIG. 23B

FIG. 24

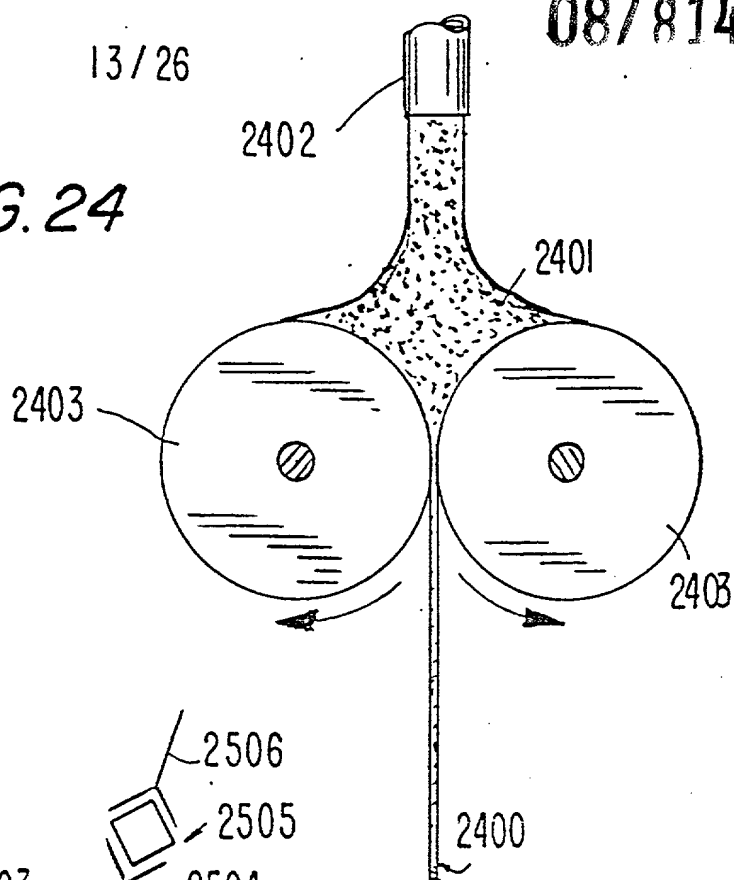


FIG. 25

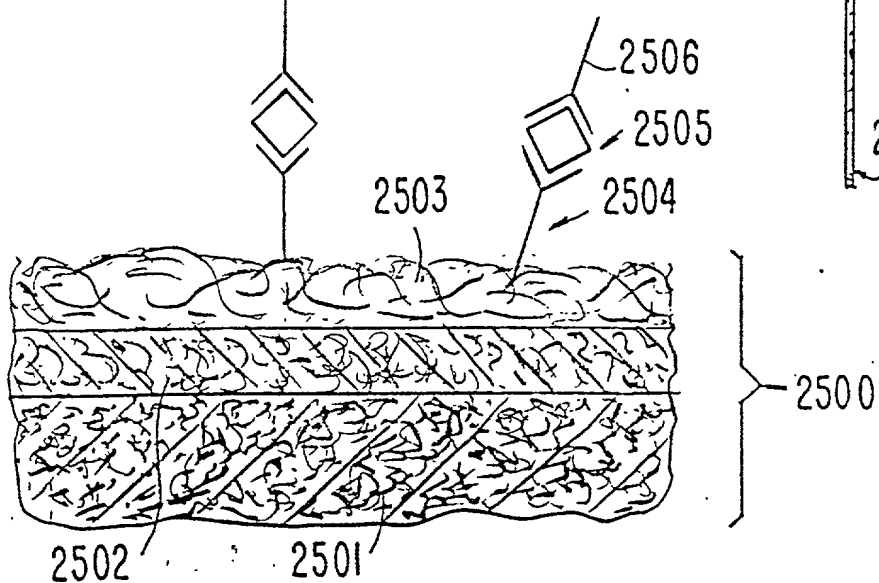


FIG. 26

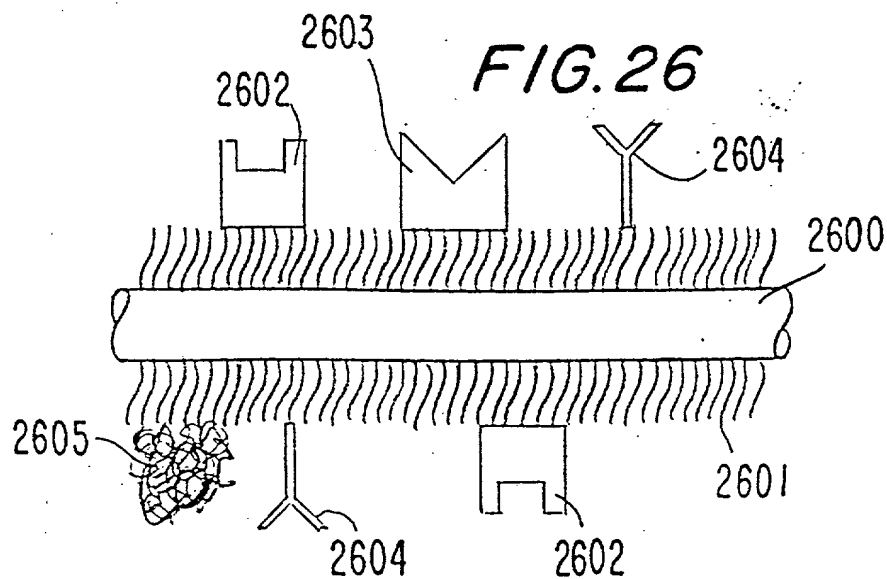


FIG. 27

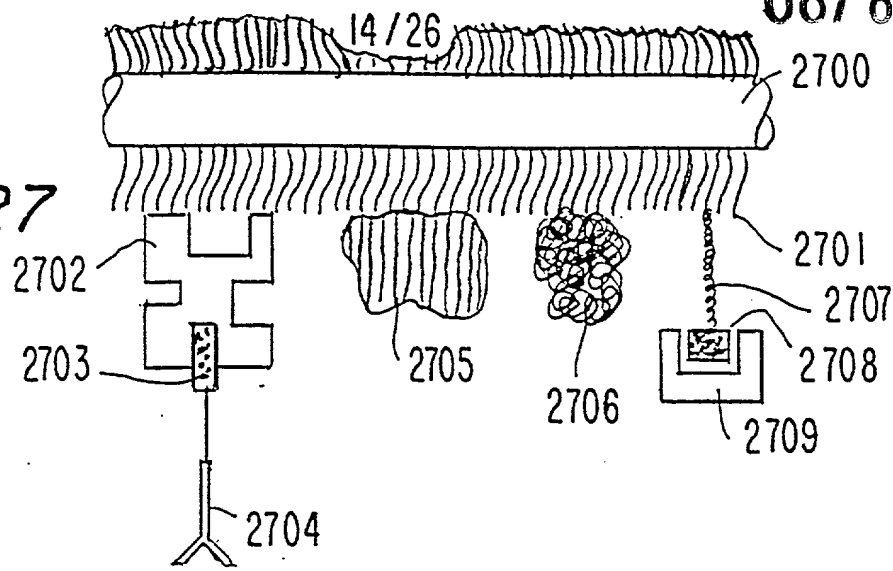


FIG. 28

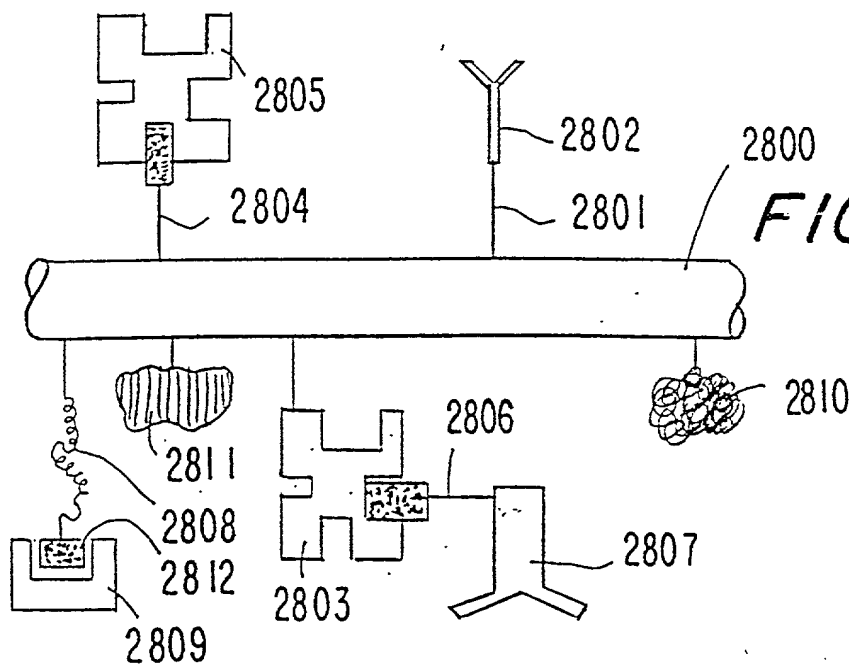
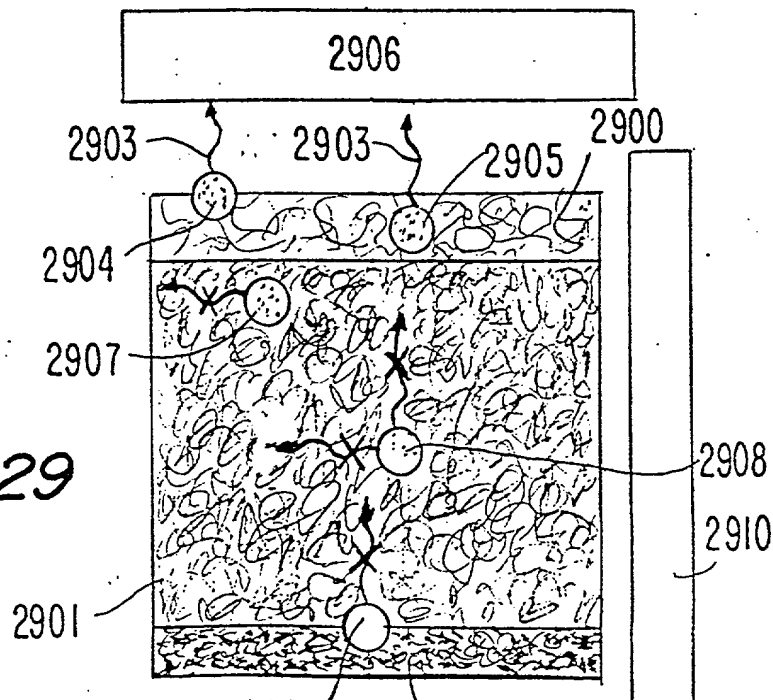


FIG. 29



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FIG. 30A

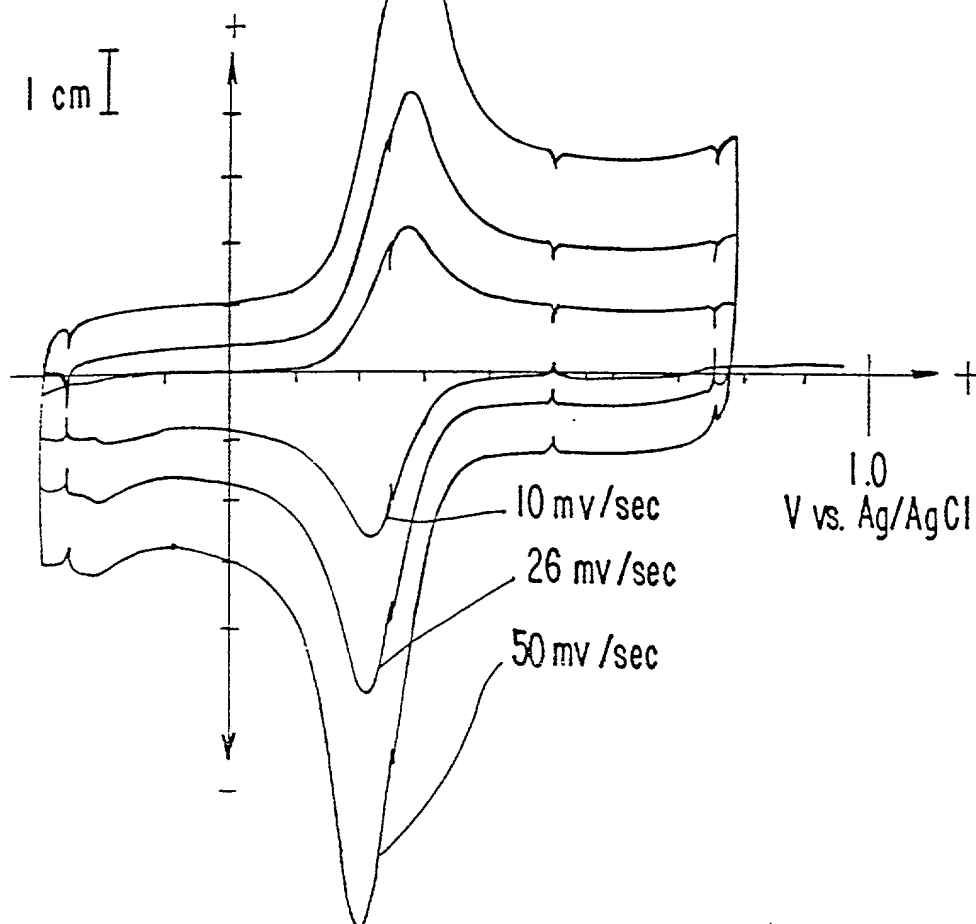


FIG. 30B

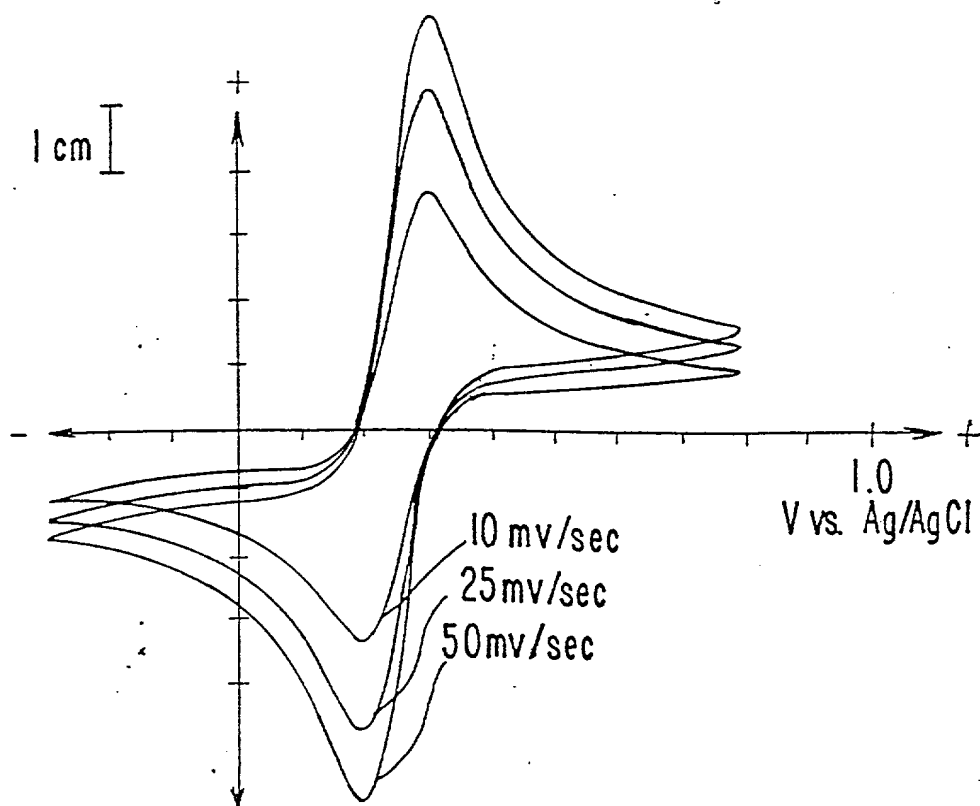


FIG. 31

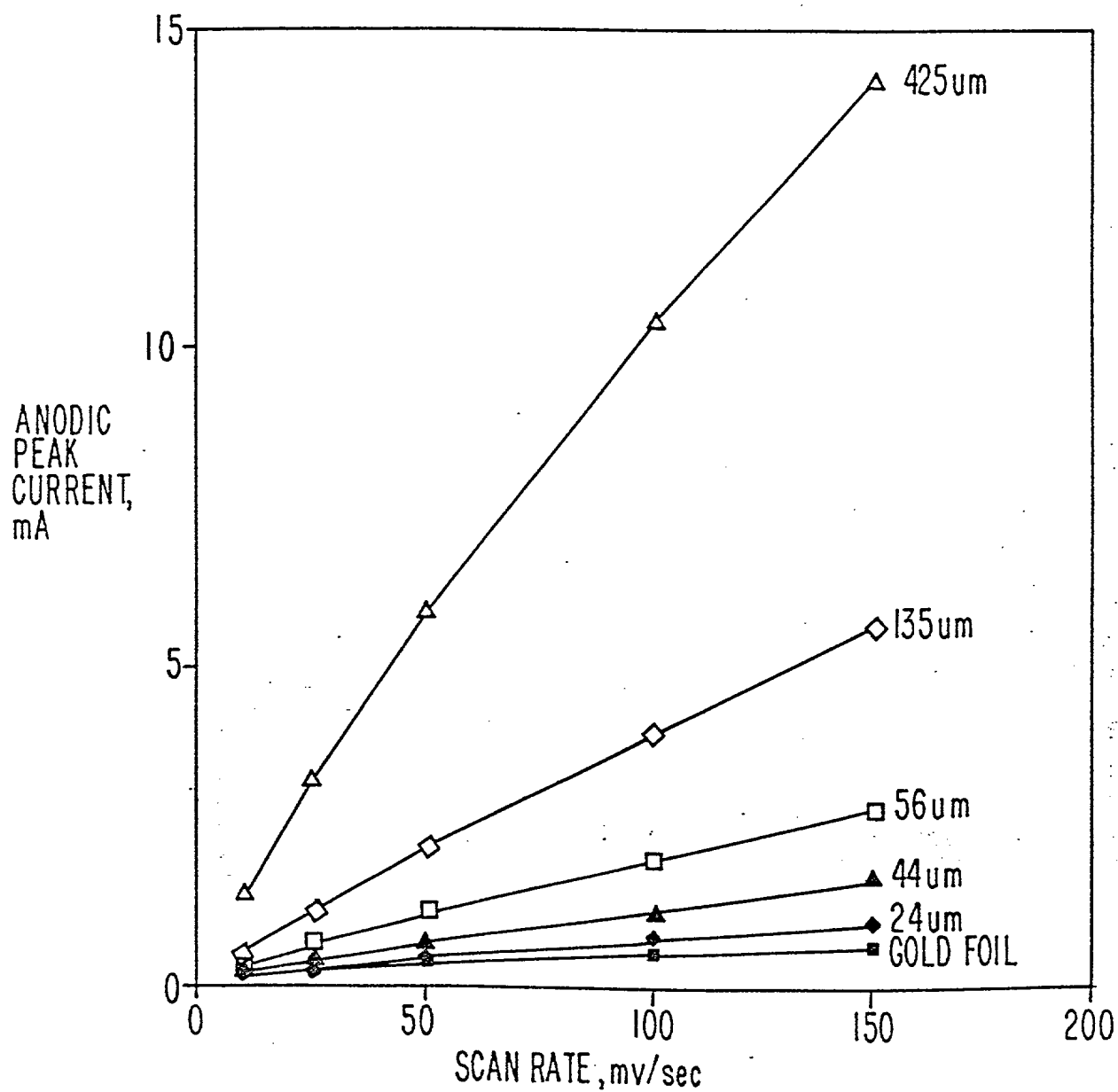


FIG. 32

ECL PEAK INTENSITY

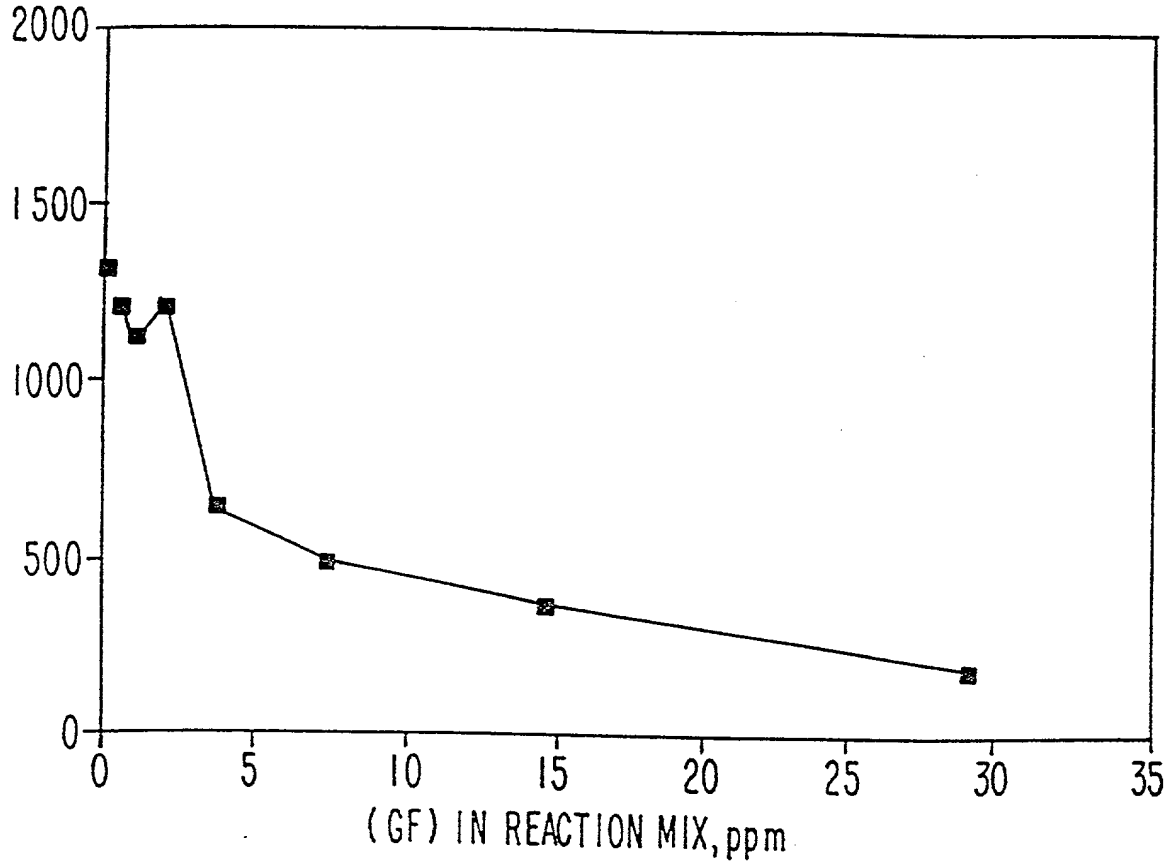
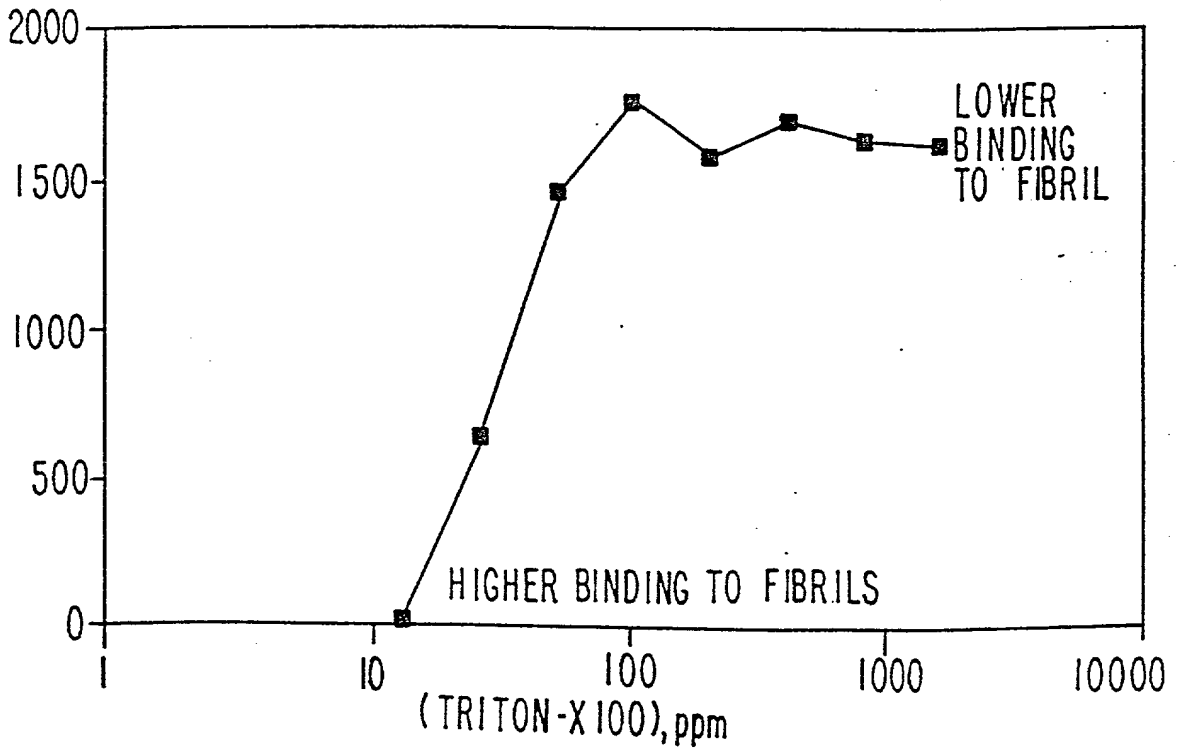
ECL PEAK INTENSITY
OF SUPERNATANT

FIG. 33



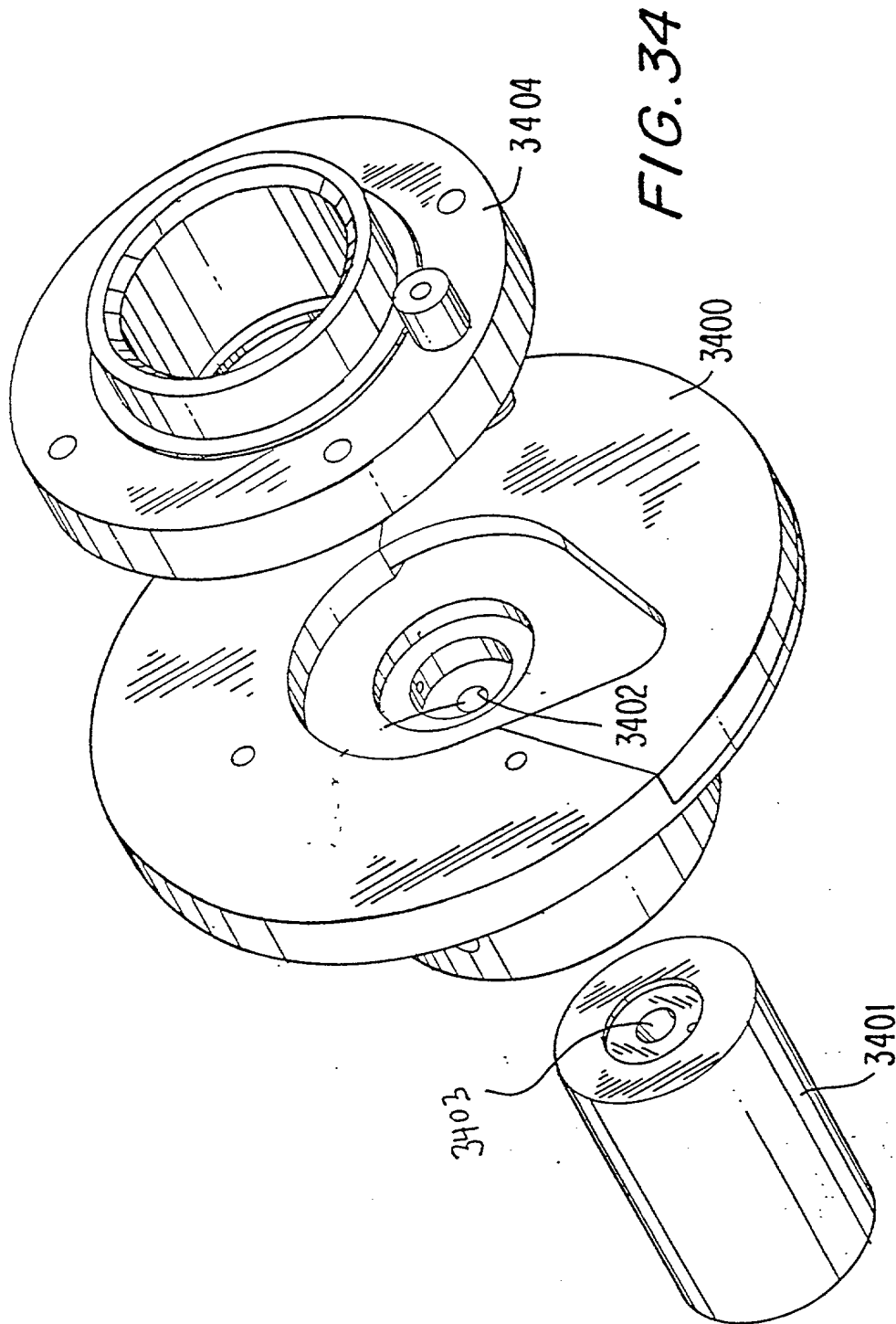


FIG. 35

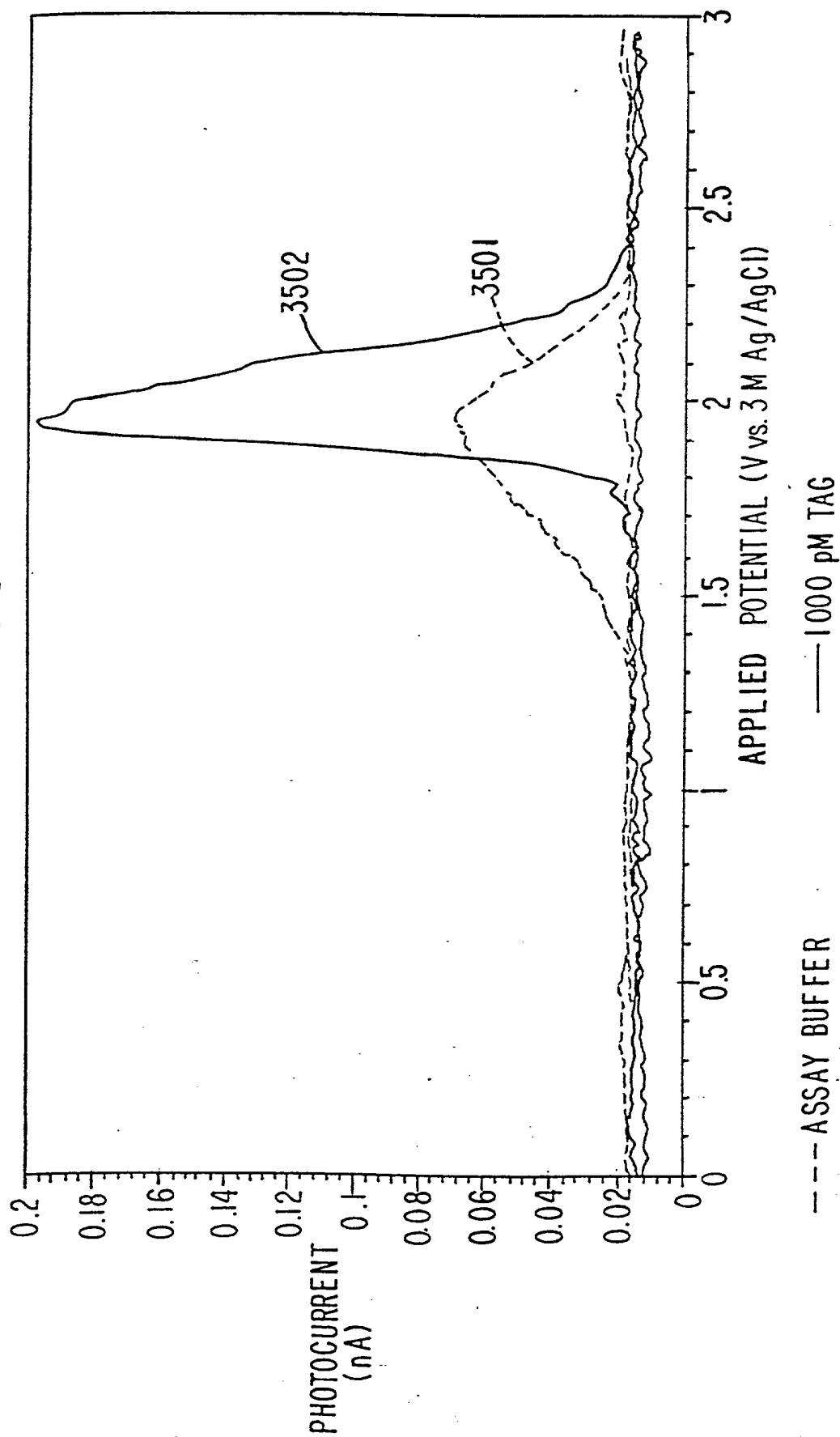


FIG. 36

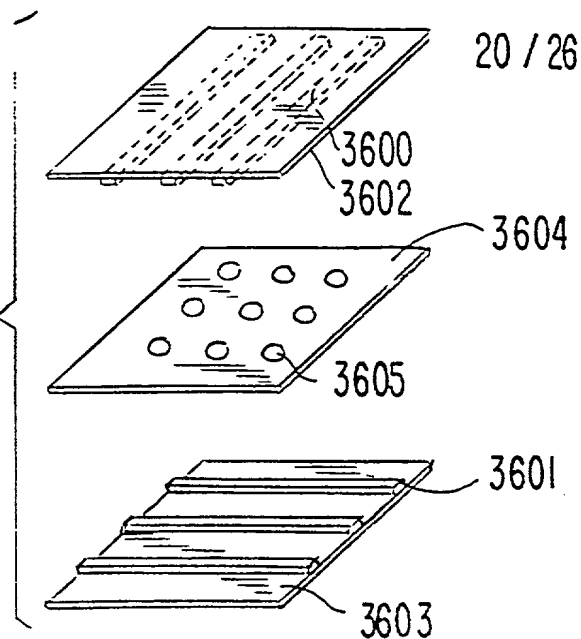


FIG. 37

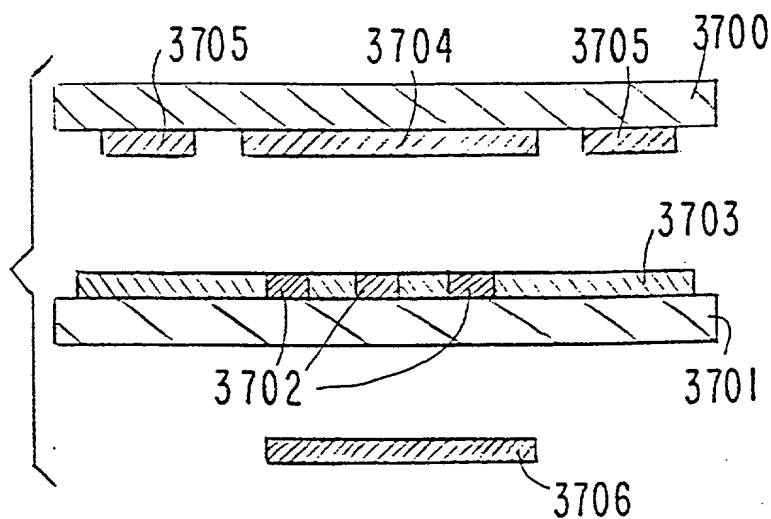


FIG. 38

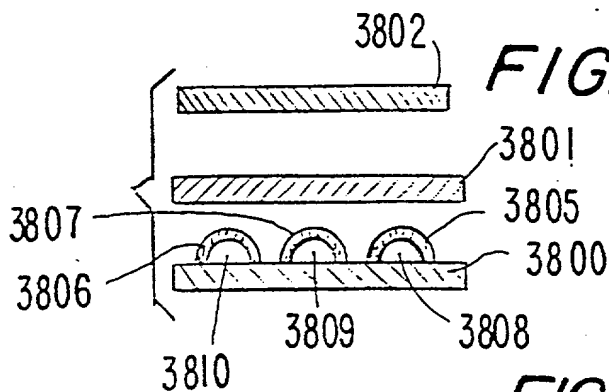


FIG. 39

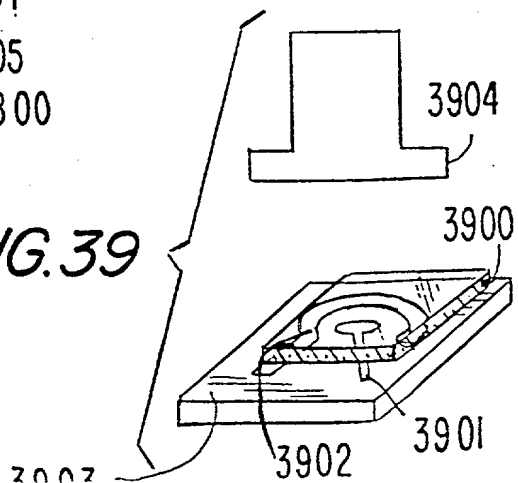


FIG. 40

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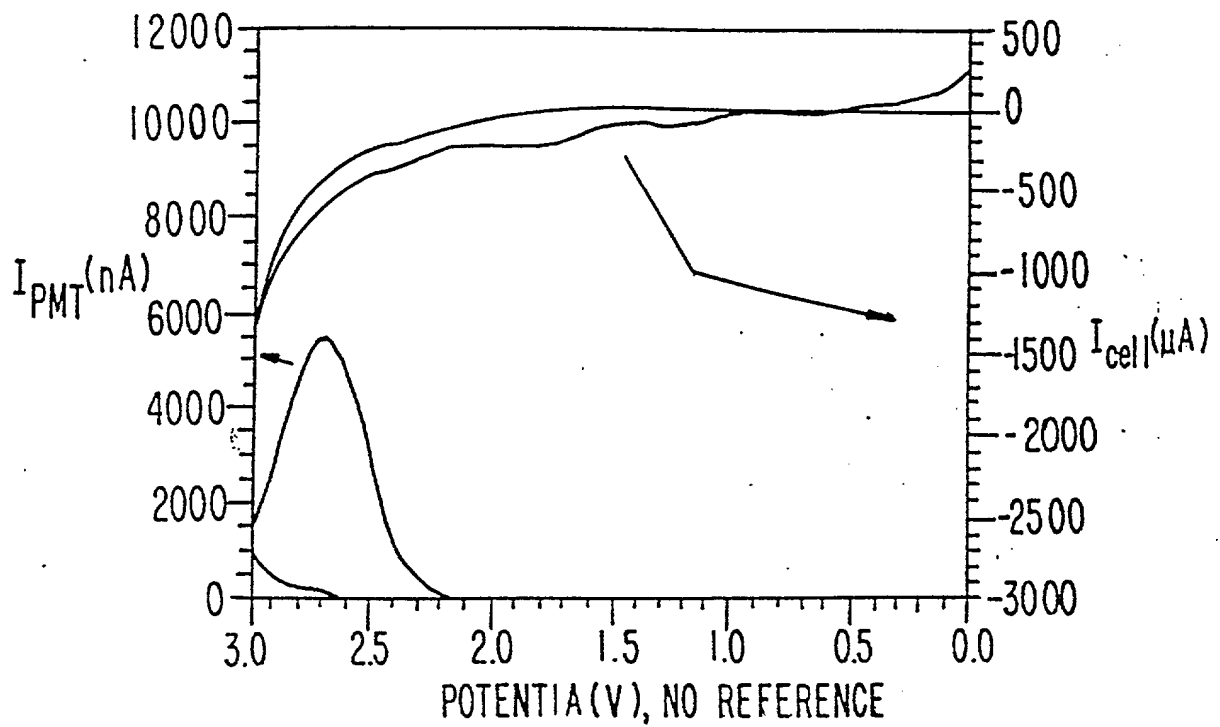
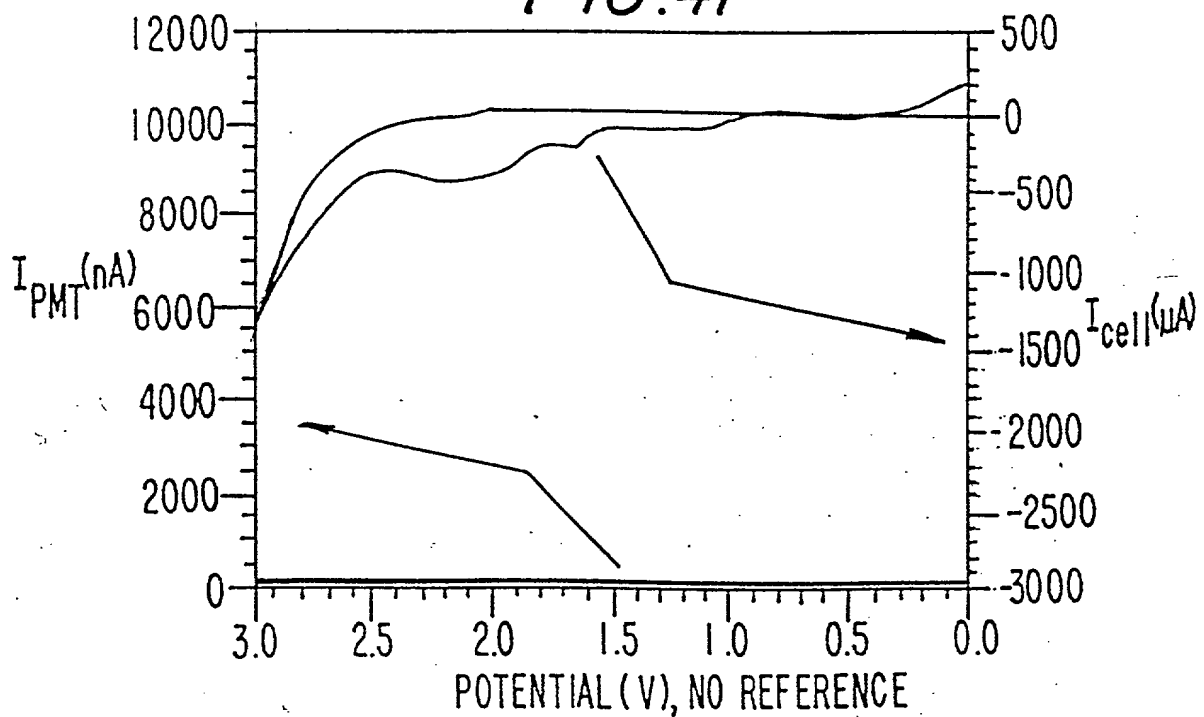


FIG. 41



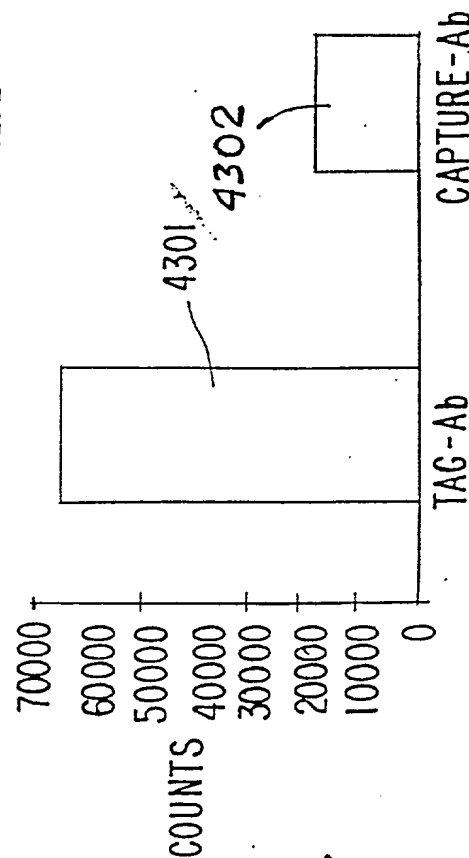
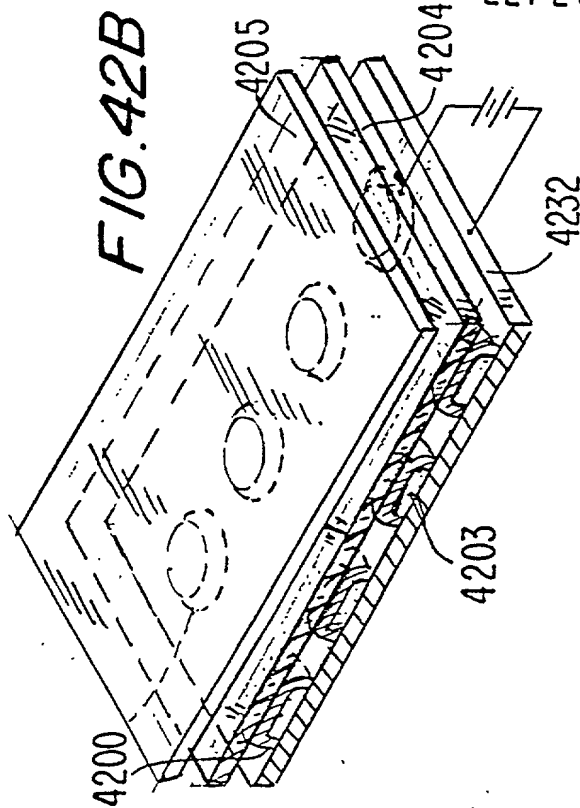
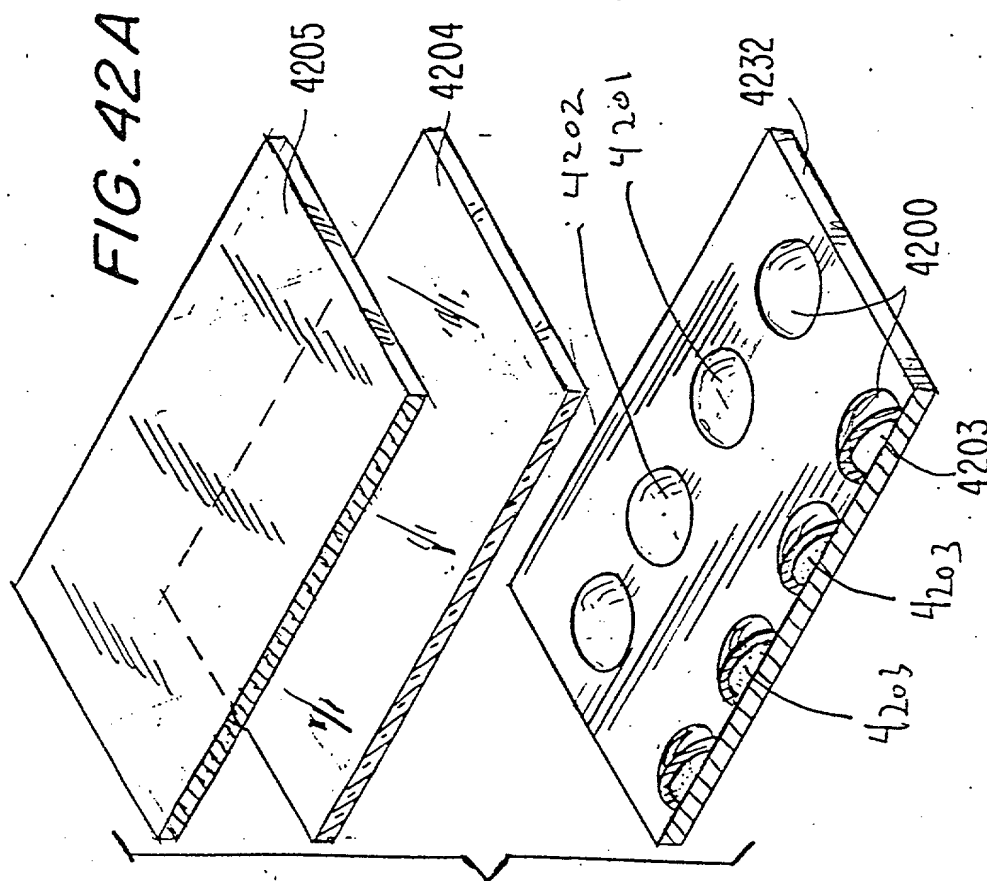


FIG. 43

FIG. 44A

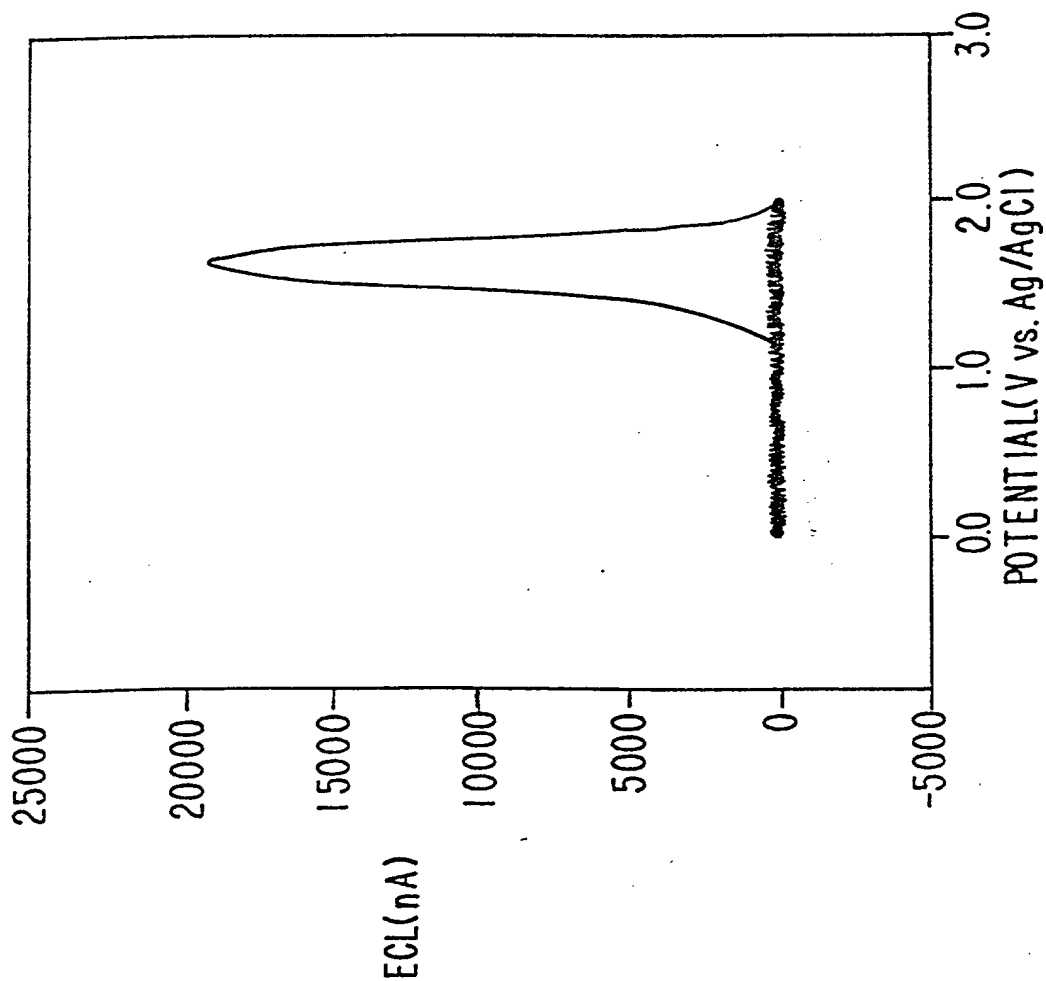
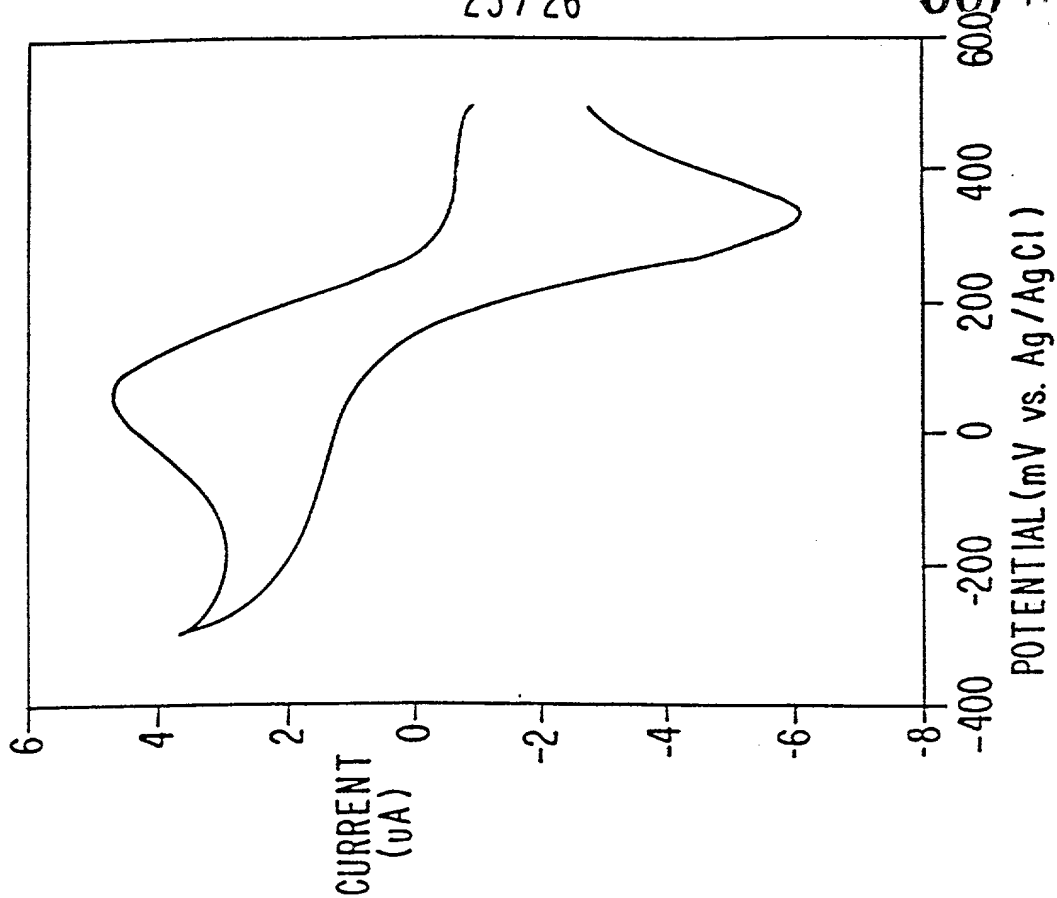


FIG. 44B



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FIG. 45A

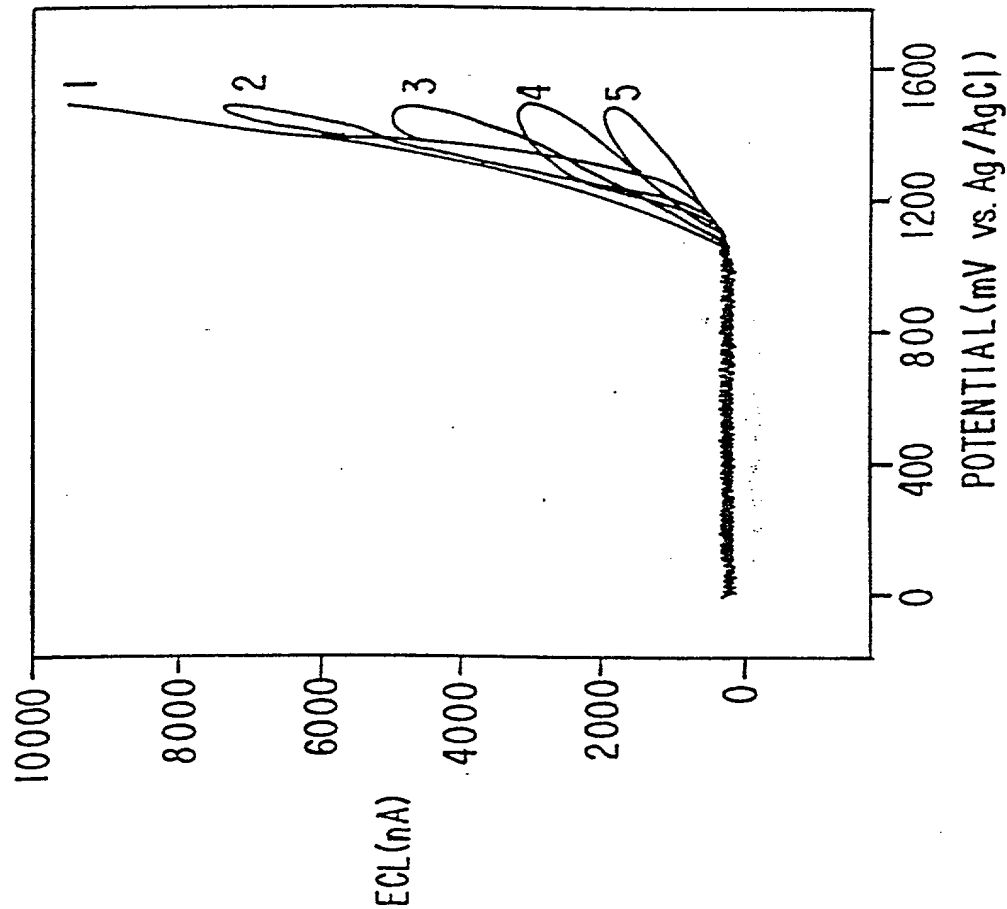


FIG. 45B

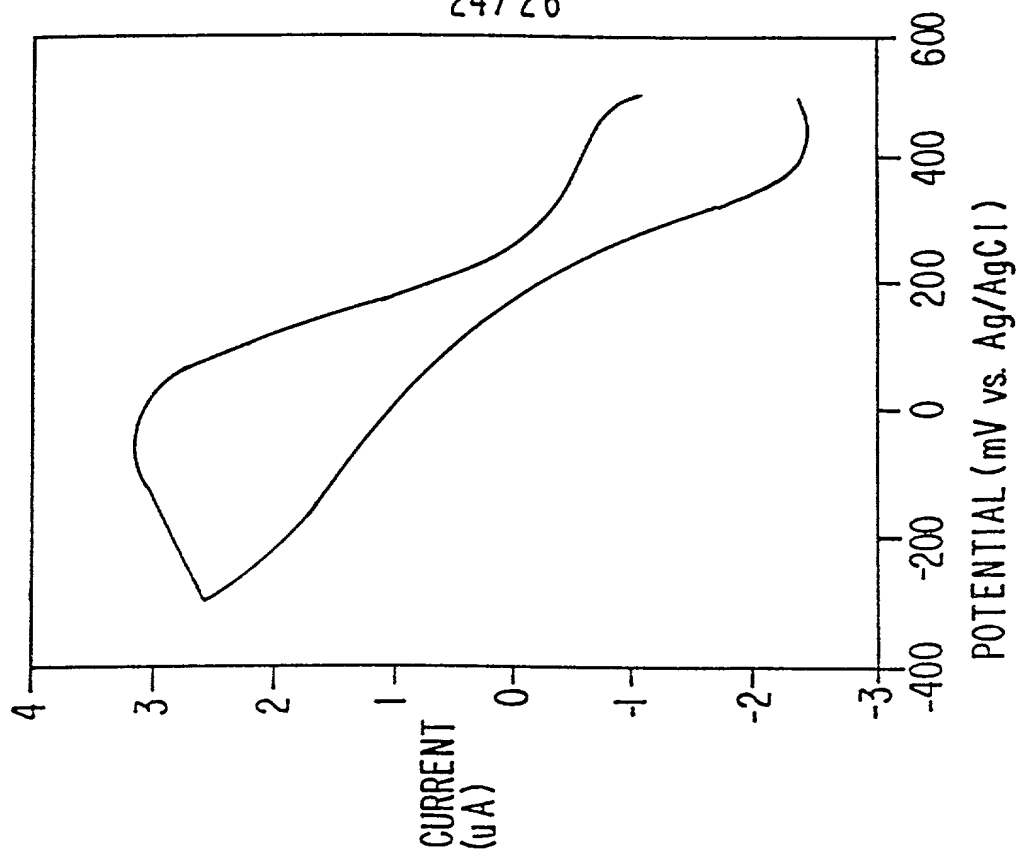


FIG. 46A

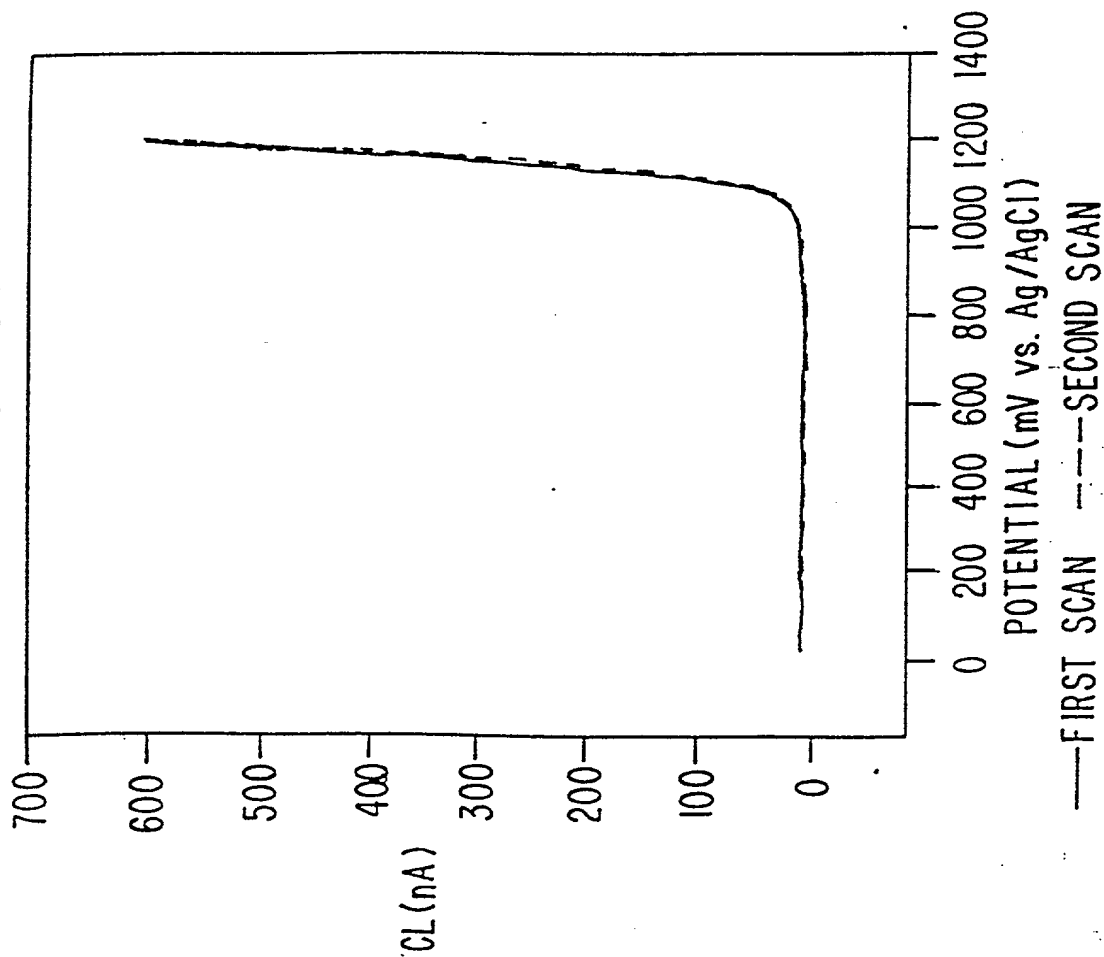


FIG. 46B

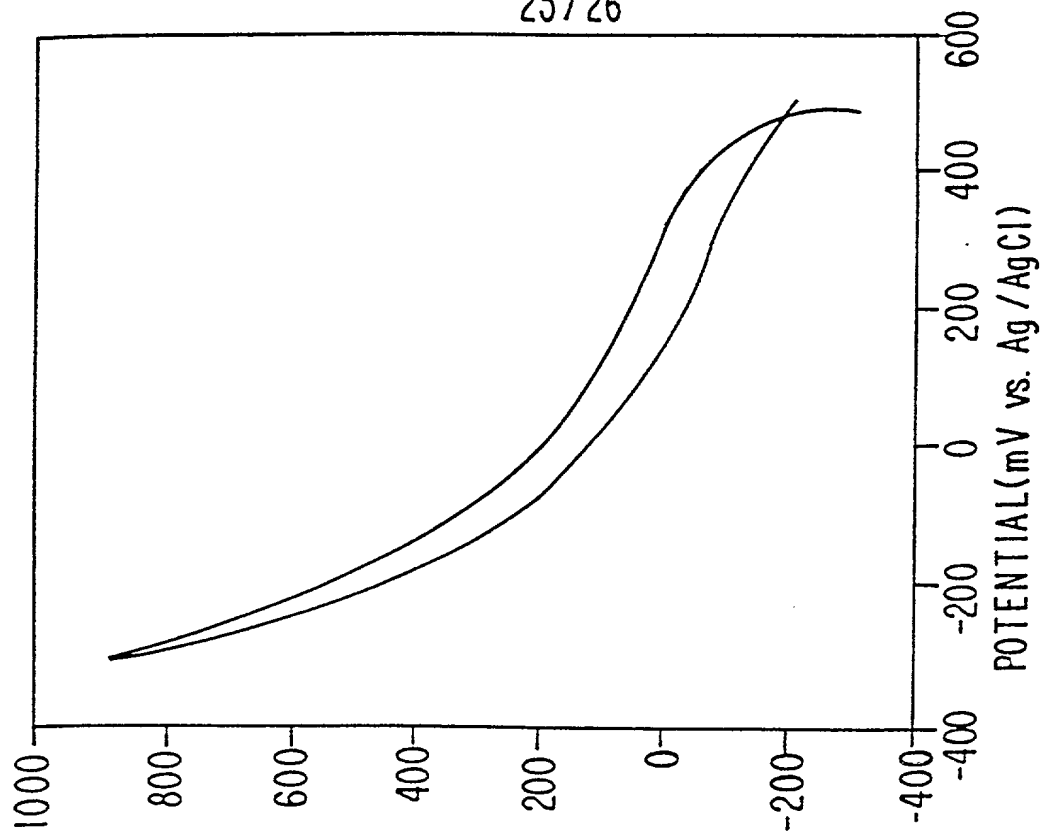


FIG. 47

